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ABSTRACT

The original aim of this project was to develop pharmacologic interventions that, when presented together with traumatic memory reactivation, could serve as novel treatments for posttraumatic stress disorder (PTSD). Candidate drugs were tested in animals in a single-trial, fear conditioning paradigm. Some of those found promising were tested in human subjects with PTSD in a single reactivation session, psychophysiological experiment employing script-driven imagery. One drug, viz., propranolol, was tested in a randomized clinical trial (RCT) employing six traumatic memory reactivation sessions. Positive findings in rats included the following. Post-reactivation mifepristone (a glucocorticoid receptor antagonist) blocked the reconsolidation of a conditioned fear memory; propranolol (a beta-adrenergic blocker) prevented mifepristone's effect. Post-reactivation clonidine (an alpha-2-adrenergic agonist) also blocked the reconsolidation of a conditioned fear memory. Post-reactivation rapamycin, a protein synthesis inhibitor, also blocked the reconsolidation of a conditioned fear memory. This last effect was found to be exerted through a post-synaptic mechanism, in contrast to fear conditioning (memory consolidation), which was found to be exerted through a pre-synaptic mechanism. In humans with PTSD, we failed to find that mifepristone, with or without the N-methyl-D-aspartate (NMDA) partial agonist d-cycloserine, both administered prior to traumatic memory reactivation, reduced subsequent physiological responding during script-driven imagery of the traumatic event. Finally, in the context of a first, double-blind, placebo-controlled, RCT, we found that a series of six traumatic memory reactivation sessions plus propranolol was efficacious in reducing symptoms in chronic PTSD. *This last finding represents a new, translational treatment for this disorder.*

1. INTRODUCTION

The original aim of this project was to develop pharmacologic interventions that, when presented along with traumatic memory reactivation, could serve as novel treatments for PTSD. The underlying theory was that candidate drugs, when given at the time of reactivation of a conditioned fear response in animals, or a traumatic memory in humans, would reduce the subsequent strength of the conditioned response or traumatic memory. We planned to test such drugs, either alone or in combination, for their possible reconsolidation-blocking properties in a hierarchy of experiments. Drugs that showed promise at a given stage of investigation were to be advanced to the next stage. In Stage I, we evaluated the ability of candidate drugs to reduce freezing in a Pavlovian cue-conditioned fear paradigm in rats. In Stage II, we evaluated the ability of candidate drug to reverse fear conditioning-induced synaptic enhancement in rat amygdala slices using whole-cell electrophysiologic recording. In Stage III, we tested the ability of a single session of candidate drug plus memory reactivation to reduce subsequent psychophysiologic responding during script-driven imagery of the traumatic event in PTSD subjects. In Stage IV, we tested the ability of traumatic memory reactivation plus candidate drug therapy sessions to reduce symptoms in PTSD patients.

The animal reconsolidation experiments entailed three phases: 1.) single-trial fear conditioning; 2.) administering the candidate drug and presenting the conditioned stimulus (reactivation); 3.) measuring the conditioned response in test trials, followed in certain cases by sacrificing the animal for electrophysiologic measurements. If a drug is an amnestic (i.e., reconsolidation-blocking) agent, the test conditioned response should be reduced in animals that previously received the active drug. In PTSD subjects, because the (past) traumatic event itself represents the (phase 1) conditioning event, the human experiments only entailed the last two phases, viz., 2.) single or multiple sessions of candidate drug along with traumatic memory reactivation; and 3.) measuring a.) psychophysiologic responses during script-driven imagery of the traumatic event, and/or b.) PTSD symptoms.

In order to rule out the possibility that nonspecific drug effects account for any findings, all experiments included vehicle/placebo control groups. Some of the experiments also incorporated non-reactivation (NR) drug control groups as well.

2. BODY

2.1. Animal work

2.1.1. Massachusetts General Hospital (MGH)

2.1.1.1. Postreactivation mifepristone (MIF) and propranolol (PROP)

2.1.1.1.1. Introduction. This study explored the potential of post-reactivation mifepristone as a novel treatment for PTSD by testing whether this drug can block reconsolidation of cue-conditioned fear in rats. Additionally, mifepristone was tried with and without concurrently administered propranolol, in order to explore whether the combination of these two drugs would have stronger reconsolidation-blocking effects than either drug alone. In PTSD, cue and context are usually not so easily separated as they can be in animal research. For example, a Vietnam veteran may be more likely to become distressed at the sight of an Asian male (cue) at night (context). For this reason, unlike in many animal studies, the rats underwent conditioning, reactivation, and testing in the same experimental chamber.

2.1.1.1.2. Methods. These are described in detail in an attached publication with 3 figures and 24 references (Pitman et al, 2011).[1] Briefly, equal numbers of male and female Sprague-Dawley rats were studied. In the first experiment designed to measure post-reactivation long-term memory (PR-LTM), rats were assigned to one of four groups of 12 rats

each: vehicle, MIF 30mg/kg, PROP 10 mg/kg, and MIF+PROP. In a follow-up experiment designed to measure non-reactivation long-term memory (NR-LTM), a fifth group received MIF without the CS (non-reactivation) on Day 2. In a second follow-up experiment designed to measure post-reactivation short-term memory (PR-STM), a sixth group was tested 4 hours after the Day 2 MIF injection.

On each experimental day, rats were placed in the chamber for 2 min. Then a tone conditioned stimulus (CS) was presented for 30 sec. Duration of freezing in response to the CS served as the conditioned response (CR). On Day 1 (conditioning), rats were trained with single presentation of the CS followed by a 1-sec 0.75mA shock unconditioned stimulus (US) immediately following tone offset. On Day 2 (reactivation), the CS was presented once without the US (reactivation). Immediately thereafter the rats were removed from the testing chamber and injected with post-reactivation (PR) drug. Drugs were not administered on any other day. However, some rats on Day 2 received non-reactivation (NR) mifepristone without being placed in the chamber. On Days 3 and 10 (test days 24 hr and 1 week after reactivation respectively), the CS was again presented without the shock, and the CR was calculated as a measure of PR-LTM. (Here "long-term" means at least one day following memory reactivation.) On Day 11 ((reinstatement), the US was presented once in the absence of the CS. On Day 12 (test), the CS again was presented once without the shock, and the CR was calculated as a measure of post-reinstatement PR-LTM. The raw dependent measure, i.e., the CR, consisted of percent freezing during each CS presentation. Percent freezing was calculated as the measured seconds of freezing divided by the 30-second maximum measurement time, multiplied by100. Freezing *decrease scores* were calculated from these raw data by subtracting a rat's CR on test days 3, 10, and 12 from its CR on Day 2 (A negative value would represent an increase over Day 2). Because the greater the freezing decrease score, the greater the loss of the fear memory following the intervention, these decrease scores represent the degree of amnesia for the conditioned CS-US association. Decrease scores were analyzed by means of repeated-measures, mixed-model analyses of variance (ANOVAs) with Gender and Group, as between-rats effects, and DAY as a repeated measure, followed by t-tests of least square means (LSMs) and differences between pairs of LSMs where appropriate.

2.1.1.1.3. Results. These are also described in detail in the attached publication (Pitman et al, 2011).[1] The following summarizes the most important results.

2.1.1.1.3.1. Gender. At virtually every day of the experiment that the CR was tested, female rats showed fewer seconds of freezing (i.e., less fear) than male rats. However, calculation of decrease scores successfully adjusted the results for this gender difference, as revealed by the absence of any significant main effects of, or interactions with, Gender in any of the results that follow.

2.1.1.1.3.2. PR-LTM. A main effect of Day indicated, across the four groups, the development of partial amnesia at Day 3, further partial amnesia by Day 10, and then reinstatement of the CR on Day 12 back to approximate Day 3 (but not back to Day 2) levels. There was also a significant MIF x PROP interaction. Results of pairwise t-tests indicated significant differences between the MIF group and each other group, such that the development of substantial amnesia occurred only with mifepristone alone, and this amnesia was paradoxically prevented by the addition of propranolol.

2.1.1.1.3.3. NR-LTM. Because only MIF alone produced significant PR-LTM (above), only MIF alone was studied. Because there was no Day 2 reactivation and hence no Day 2 CR data, decrease scores could not be calculated. Instead ANOVA was performed on the raw

freezing data, with REACTIVATION (present or absent) as a between-rat effect and DAY (3, 10) as a repeated measure. There was a significant main effect of REACTIVATION, such that only when mifepristone was preceded by memory reactivation was there a notable subsequent decrement in conditioned freezing.

2.1.1.1.3.4. PR-STM. Again only MIF alone was studied. Freezing was measured either at 4 hours (PR-STM) or at 24 hours (PR-LTM) post-reactivation. Only rats in the latter group developed amnesia. Rats in the PR-STM group showed virtually no decrease in freezing.

2.1.1.1.4. Comment. The results of this published study show that mifepristone administered systemically to rats following the presentation of a previously conditioned fear cue significantly reduced subsequent cue-induced conditioned responding, as manifest in a shorter duration of freezing. The percent reduction in percent freezing from reactivation to 24 hours post-reactivation was more than 50%, which represents approximately two-thirds of the approximate 75% reduction produced by the standard reconsolidation-blocking route and drug, viz., intra-amygdala anisomycin,[2] suggesting that systemic mifepristone may be nearly as efficacious a reconsolidation blocker as intra-amygdala anisomycin, at least under the circumstances of our study. Our design incorporated controls necessary to infer that reconsolidation blockade was the mechanism behind this effect. First, the (partial) amnesia for the CS-US association induced by post-reactivation mifepristone was relatively long-lasting (for rats), viz., a week, i.e., there was no evidence of spontaneous recovery. Second, the CR was (partially) reinstated by readministration of the shock US in the absence of the tone CS Third, non-reactivation mifepristone, i.e., drug in the absence of memory reactivation, produced no amnesia. Fourth, when measured four hours following post-reactivation mifepristone, the CR was still fully present, whereas it was reduced the next day. Like consolidation, reconsolidation is a time-dependent process that affects long- but not short-term, memory.

Our results further suggest that post-reactivation systemic mifepristone is worth exploring in human reconsolidation blockade studies, including as a potential novel treatment for PTSD. A paradoxical result was that concurrent post-reactivation propranolol prevented the memory reconsolidation-blocking effect of mifepristone. Propranolol is known to antagonize the memory consolidation-*enhancing* effect of corticosterone by blocking a final common pathway of hormonal modulation of memory, viz., noradrenergic innervation of the basolateral amygdala.[3] However, it has been found that basolateral amygdala lesions block not only the memory consolidation-enhancing effect of the glucocorticoid agonist RU28362 (administered intrahippocampally) on inhibitory avoidance, but also the memory-reducing effect of mifepristone.[4] Similar results have been obtained with intra-amygdala beta-blockade (Roozendaal B, personal communication of unpublished data). Our results extend these findings to reconsolidation, in that we found that systemic propranolol blocked the reconsolidation-*reducing* effect of mifepristone. This finding suggests that a permissive level of (nor)adrenergic activity is required not only for the memory-enhancing effects of glucocorticoids but also for the memory-reducing effects of their antagonists. The mechanism of this permission remains to be elucidated. From a translational standpoint, the finding that propranolol prevents rather than enhances the reconsolidation-blocking effect of mifepristone, at least in the doses used in our study, militates against attempting to combine these two drugs in a reconsolidation-blockade treatment approach to PTSD.

In our study, systemic propranolol alone had only a small, not statistically significant, reconsolidation-blocking effect on conditioned fear. This negative result is partially at odds with results of some previously published studies that used the same 10 mg/kg dose as in our study.[5-7] or nearly the same dose (5 mg/kg) [8] The discrepancy might be explained by design and

methodological differences. Our study used a cue conditioning procedure, whereas one of those previous positive studies employed inhibitory avoidance,[7] and one employed context conditioning.[8] Of the two studies reporting that propranolol blocked reconsolidation of cue conditioning, one[6] used Long Evans, rather than Sprague Dawley rats as herein. In both cue-conditioning studies,[5;6] the conditioned responses were acquired in one experimental chamber (context), but reactivated and then tested in another chamber. For reasons of clinical applicability described above, in our study all procedures were performed in the same chamber.

Interestingly, in the last of the two above cited studies,[6] propranolol failed to block the reconsolidation of inhibitory avoidance, whereas systemic mifepristone had previously succeeded in doing so in a study from the same laboratory.[9] In addition to the our results, this suggests that, compared to propranolol, mifepristone may be a superior reconsolidation blocker of conditioned fear across various designs and may ultimately turn out to be a more useful treatment for PTSD. At any rate, results of translational studies in animals can only identify effects that deserve further investigation in humans; one-to-one correspondence is not assured.

This study has several limitations. For reasons discussed in the introduction, CRs were only tested in a single context (chamber). Consequently, renewal could not be assessed, and the possibility that context conditioning played some role in the observed results cannot be completely ruled out. However, as noted above, any freezing that resulted from the rats being placed in the conditioning context was far below the level of freezing induced by the tones and had ended long before the tones were played, supporting the conclusion that the observed cue-induced freezing was independent of any context-induced freezing.

A second limitation is that our design employed only single doses of mifepristone (30 mg/kg) and propranolol (10 mg/kg). These doses were chosen on the basis of their having most often been used in relevant published rat studies, and the consideration that higher doses on a translational mg/kg basis could be prohibitive in humans. The possibilities that different doses of each drug might produce greater reconsolidation blockade, and that different doses of the two drugs in combination might block reconsolidation cannot be ruled out.

A third limitation is the possibility that the mifepristone-propranolol interaction observed in our study was pharmacokinetic rather than pharmacodynamic in nature. In other words, one of the drugs may have increased or decreased metabolism of the other, thereby affecting blood levels. However, this explanation is unlikely given that such a pharmacokinetic interaction has not been previously reported and that the metabolism of mifepristone and propranolol rely upon different cytochrome P450 enzymes.

Finally, the mifepristone results obtained in this study have been interpreted within the framework of glucocorticoid receptor blockade. However, this drug has other, especially anti-progesterone, properties which could partially underlie its observed effect. Because mifepristone is currently the only suitable glucocorticoid receptor blocker approved for human use, this limitation was unavoidable. Although the underlying mechanism of action is of scientific interest, it may not be of great concern from a clinical standpoint. The primary objective of our study was to test reconsolidation-blockers as potential candidates for treating PTSD, regardless of their mechanisms of action.

2.1.1.2. Postreactivation oxytocin

2.1.1.2.1. Introduction. Oxytocin has been found in animal studies to reduce the consolidation of conditioned fear memories, but it has not been reported to reduce their reconsolidation.[10] This as yet unpublished study explored the potential of post-reactivation

oxytocin as a novel treatment for PTSD by testing whether this drug can block reconsolidation of cue-conditioned fear in rats.

2.1.1.2.2. Methods

2.1.1.2.2.1. Rats. Equal numbers of male and female Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing approximately 250 g were co-housed (two of the same gender per cage) at the Massachusetts General Hospital Center for Comparative Medicine in transparent polyethylene cages and maintained on a 12-hr light/dark schedule with free access to food and water. They were transported to our laboratory for the study's procedures in the early afternoon and returned to the housing facility at the end of each day. On each of the two days prior to the experiment, rats were handled for five minutes and then placed in the conditioning chamber for five minutes of habituation. Each experimental Plexiglas chamber (Coulburn Instruments, Whitehall, PA) measured 25 x 29 x 29 cm and was situated inside a sound-attenuated box (Med Associates, Burlington, VT).

2.1.1.2.2.2. Drugs. Oxytocin powder, 50IU/mg (Sigma, St Louis, MO), in the amounts of either 0, 0.0125, 0.3125, or 2.5 mg was dissolved in 0.5 ml normal saline to prepare four doses, as follows: 0 mg/kg (vehicle), 0.05 mg/kg, 1.25 mg/kg, and 10 mg/kg for subcutaneous injection. (The rats used in this research weigh approximately 0.25 kg.)

2.1.1.2.2.3. Procedures. On each experimental day, rats were placed in the chamber for 2 min. Then a 4-kHz, 80 dB SPL tone (conditioned stimulus, CS) was presented for 30 sec. Duration of freezing served as the CR and was measured via motion-sensing computer software (FreezeScan, Clever Systems, Reston, VA). When first placed in the experimental chamber, some rats were observed to show a small degree of immobility, but this did not last more than 10 sec. as estimated by observation in any rat at any test, usually much less. This means that all rats regained their mobility at least 110 sec. prior to the tone presentation, indicating that any incidental freezing to the context did not overlap freezing to the tone cue. On Day 1, rats were trained with single presentation of the CS followed by a 1-sec 0.75mA shock (US) that was delivered via the grid floor immediately following tone offset. The rats then remained in the chamber for 1 min. and then returned to their home cages. On Day 2 the CS was presented once without the US (reactivation). Immediately thereafter the rats were removed from the testing chamber and injected with post-reactivation (PR) drug. Drugs were not administered on any other day. On Days 3 and 10 (24 hr and 1 week after reactivation respectively), the CS was again presented once without the shock, and the CR was calculated as a PR-LTM. On Day 11 the US was presented once in the absence of the CS (reinstatement). On Day 12, the CS again was presented once without the shock, and the CR was calculated as a measure of post-reinstatement PR-LTM. There were four PR drug groups corresponding to the four oxytocin dose levels. Each group consisted of 12 male and 12 female rats.

2.1.1.1.2.4. Data analysis. The raw dependent measure consisted of percent freezing during each CS presentation, i.e., the CR. Freezing *decrease scores* were calculated from these raw data by subtracting the rat's CR on test days 3, 10, and 12 from the rat's Day 2 CR (a negative value would represent an increase over Day 2). Decrease scores were analyzed by means of repeated-measures, mixed-model analyses of variance (ANOVAs) with Gender and oxytocin dose as between-rats effects, and DAY as a repeated measure, followed by t-tests of least square means (LSMs) and differences between LSMs where appropriate.

2.1.1.2.3. Results

2.1.1.2.3.1. Across doses and days, female rats showed fewer seconds of freezing (i.e., less fear) than male rats. However, calculation of decrease scores successfully adjusted

the results for this gender difference, as revealed by the absence of any significant main effects of, or interactions with, Gender in any of the results that follow.

2.1.1.2.3.2. PR-LTM. Figure 2.1.1.2.3.2 displays mean raw CRs as percent freezing for each of the four oxytocin dosage groups on each test day collapsed across gender. Table 2.1.1.2.3.2 displays freezing decrease scores for each group. In the analysis of these decrease scores, there was a significant main effect of DAY: $F(2,282)=10.8$, $p<0.0001$, LSMs with standard errors (SEs) in parentheses were 24-hr PR-LTM: 6.9 (2.1), $p<0.001$; 1-week PR-LTM: 17.3 (2.1), $p<0.001$, post-reinstatement PR-LTM: 4.7 (2.1), $p=0.02$. Results of pairwise t-tests indicated significant differences between 24-hr PR-LTM vs. 1-week PR-LTM, and 1-week PR-LTM vs. post-reinstatement PR-LTM, but not between 24-hr PR-LTM vs. post-reinstatement PR-LTM. These results indicate the development of amnesia at 24 hrs post-reactivation, further amnesia at 1-week post-reactivation, and then reinstatement of the CR back to approximate 24-hr post-reactivation levels (but not back to reactivation levels). There was also a significant main effect of Dose, $F(3,282)=14.4$, $p<0.0001$; LSMs with SEs in parentheses were 0.00 mg/kg 1.0 (2.4), p=ns; 0.05 mg/kg 2.8 (2.4), p=ns; 1.25 mg/kg 18.0 (2.4), $p<0.001$; 10.00 mg/kg 1.0 (2.4),

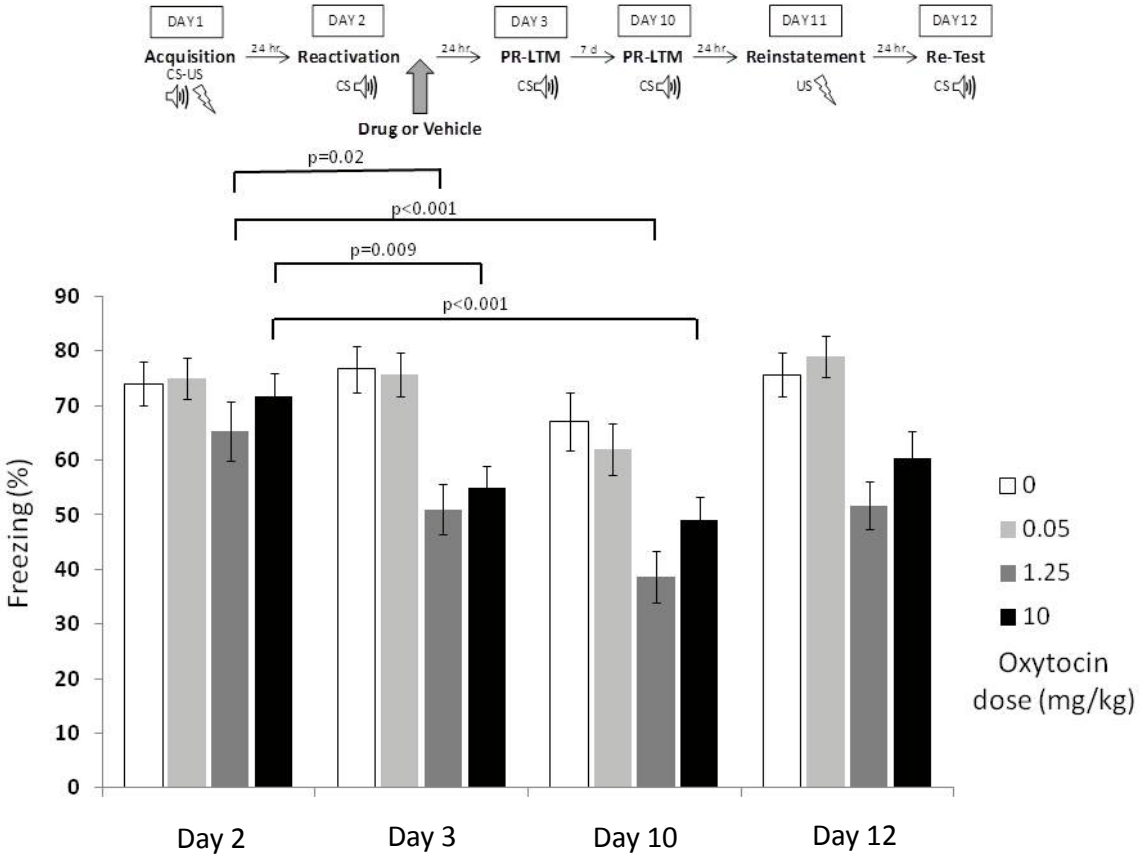

Figure 2.1.1.2.3.2 Post-reactivation long-term memory (PR-LTM) in the four dosage groups. Shown are group mean percent freezing to the tone (i.e., conditioned fear response) collapsed across gender on Day 2 (reactivation prior to drug), Days 3 and 10 (test days), and Day 12 (test day following reinstatement). Bars=standard error.

Oxytocin Dose (mg/kg)	Day 3 1 day Post-Reactivation	Day 10 1 week Post-Reactivation	Day 12 1 day Post-Reinstatement
0.00	-2.5 (3.7)	7.0 (5.3)	-1.7 (1.1)
0.05	-0.7 (3.6)	13.0 (4.4)	-4.0 (3.8)
1.25	14.3 (4.5)	26.6 (4.5)	13.7 (4.7)
10.00	16.7 (2.7)	22.6 (4.5)	11.0 (3.4)

Notes: Displayed are decreases in the conditioned response (percent freezing) from Day 2. (Figures in parentheses are SEs.)

Table 2.1.1.2.3.2 Reduction in Post-Reactivation Long-term Memory at Various Oxytocin Doses

16.7 (2.4). Results of pairwise t-tests indicated significant differences between both 0.00 mg/kg and 0.05 mg/kg on the one hand and 1.25 mg/kg and 10.00 mg/kg on the other. These results indicated that as the oxytocin dose became larger, PR-LTM decreased, although though there was no further decrease between the 1.25 mg/kg and 10.00 mg/kg doses. The Day x Dose interaction was not significant.

2.1.1.2.4. Comment. These results show for the first time that oxytocin administered systemically to rats following the presentation of a previously conditioned fear cue significantly reduces subsequent cue-induced conditioned responding, as manifest in a shorter duration of freezing. The largest reduction in percent freezing (27%) occurred in the 1.25 mg/kg group at one-week post-reactivation and was less that that induced by mifepristone (above). The (partial) amnesia for the CS-US association induced by post-reactivation mifepristone was relatively long-lasting (for rats), viz., a week, i.e., there was no evidence of spontaneous recovery. As with mifepristone (above), reinstatement of the CR in rats that had received the higher oxytocin doses was only partial, with some amnesia remaining.

This study has several limitations. Importantly, the absence of two important controls, i.e., non-reactivation oxytocin, and testing for PR-STM, precludes a firm conclusion that reconsolidation blockade is the mechanism by which PR oxytocin reduces conditioned fear memory. These controls need to be incorporated into further research with oxytocin. As with mifepristone, CRs were only tested in a single context (chamber).

2.1.1.3. Postreactivation nabilone

2.1.1.3.1. Introduction. Systemic administration of cannabinoid receptor agonists has been found to impair memory consolidation.[11] Their effect on reconsolidation has not been studied. This study exlored the potential of post-reactivation nabilone, a synthetic cannabinoid, as a novel treatment for PTSD by testing whether this drug can block reconsolidation of cue-conditioned fear in rats.

2.1.1.3.2. Methods

2.1.1.3.2.1. Rats. Same as in §2.1.1.2.2.1

2.1.1.3.2.2. Drugs. Nabilone (Sigma, St Louis, MO) in a dose of 0.25 mg (approximately 1 mg/kg) was dissolved in 0.5 ml propylene glycol vehicle and injected subcutaneously.

2.1.1.3.2.3. Procedures. Same as in §2.1.1.2.2.3. Again, NR nabilone was not administered, and PR short-term memory was not studied. Each of two groups and (nabilone and vehicle) consisted of 12 male and 12 female rats.

2.1.1.3.2.4. Data analysis. Same as in 2.1.1.2.2, except that the between-rats effects were Gender, Drug (vehicle vs. nabilone), and Day.

2.1.1.3.3. Results

2.1.1.3.3.1. Gender. Calculation of decrease scores successfully adjusted the results for this gender difference, as revealed by the absence of any significant main effects of, or interactions with, Gender in any of the results that follow.

2.1.1.3.3.2. PR-LTM. Figure 2.1.1.3.3.2 displays mean raw CRs as percent freezing for each group on each test day collapsed across gender. Difference scores were calculated by subtracting percent freezing on Days 3, 10, and 12 each from percent freezing on Day 2. Table 2.1.1.3.3.2 displays these freezing decrease scores for each group. In the analysis of these decrease scores, there was a significant main effect of DAY: $F(2,122)=3.4$, $p<0.03$; LSMs with standard errors SEs in parentheses were 24-hr PR-LTM (Day 3): 10.0 (3.0), $p=0.001$; 1-week PR-LTM (Day 10): 20.6 (3.0), $p<0.001$; post-reinstatement PR-LTM (Day 12): 12.0 (3.0), $p<0.001$. Results of pairwise t-tests indicated significant differences between 24- hr PR-LTM vs. 1-week PR-LTM. and 1-week PR-LTM vs. post-reinstatement PR-LTM, but not between 24-hr PR-LTM vs. post-reinstatement PR-LTM. As with mifepristone and oxytocin (above) these results indicate the development of (partial) amnesia at 24-hr post-reactivation, further amnesia at 1-week post-reactivation, and then reinstatement of the CR back to approximate 24-hr post-reactivation levels (but not back to reactivation levels). There was also a significant main effect of Drug, $F(1,122)=9.0$, $p=0.003$; LSMs with SEs in parentheses were vehicle 2.7 (0.8), and nabilone 5.9 (0.7). Results of a pairwise t-test indicated a significant difference between nabilone and vehicle. The Day x Drug interaction was not significant.

2.1.1.3.4. Comment. These results show for the first time that nabilone administered systemically to rats following the presentation of a previously conditioned fear cue significantly reduces subsequent cue-induced conditioned responding, as manifest in a shorter duration of freezing. The percent reduction in percent freezing from reactivation to one-week PR, viz., 21%, was again less that that induced by mifepristone (above). The (partial) amnesia for the CS-US association induced by post-reactivation nabilone was relatively long-lasting (for rats), viz., a week, i.e., there was no evidence of spontaneous recovery. As with mifepristone and oxytocin (above), reinstatement of the CR in rats that had received nabilone was only partial, with some amnesia remaining.

This study has the same limitations as the oxytocin study (above).

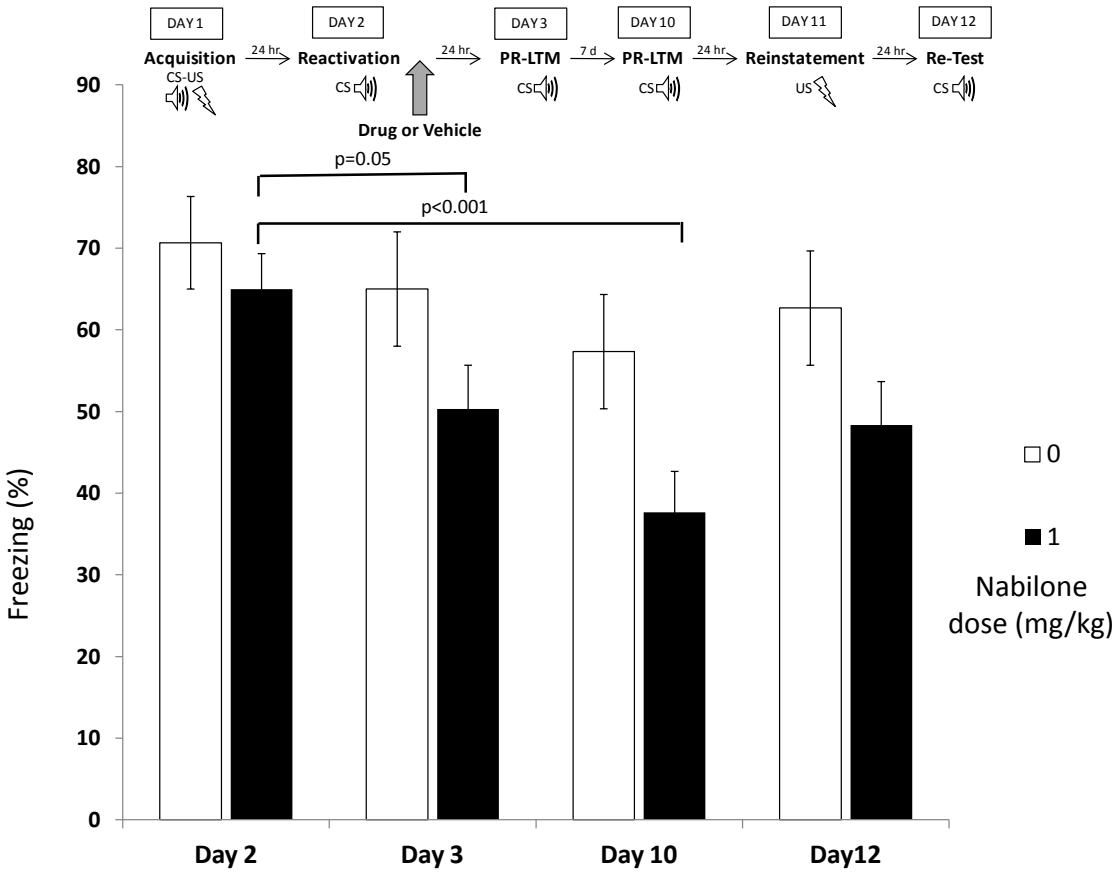

Figure 2.1.1.3.3.2 Post-reactivation long-term memory (PR-LTM) in the nabilone and vehicle groups. Shown are group mean percent freezing to the tone (i.e., conditioned fear response) collapsed across gender on Day 2 (reactivation prior to drug), Days 3 and 10 (test days), and Day 12 (test day following reinstatement). Bars=standard error.

Oxytocin Dose (mg/kg)	Day 3 1 day Post-Reactivation	Day 10 1 week Post-Reactivation	Day 12 1 day Post-Reinstatement
Vehicle	5.6 (4.7)	13.3 (4.7)	8.0 (4.7)
Nabilone	14.7 (4.0)	20.7 (4.0)	16.6 (4.0)

Notes: Displayed are decreases in the conditioned response (percent freezing) from Day 2. (Figures in parentheses are SEs.)

Table 2.1.1.3.3.2 Decrease in Post-Reactivation Long-term Memory in the Nabilone and Vehicle Groups

2.1.1.4. Additional drugs. During the course of the project, we also tried various other drugs (all approved for human use) within the protocol described above. Figure 2.1.1.4.1 presents decrease in percent freezing from Day 2 (before reactivation+drug) to Day 3 (one day after reactivation+drug) for all drugs studied in animal work at MGH. This decrease is a putative index of degree of reconsolidation blockade induced by the drug. Data in all bars except the two to the right were obtained using a 0.75 mV unconditioned stimulus (UCS). Data in the two bars to the right were obtained using a 1.5 mV UCS. Drug name abbreviations from left to right are as follows: HAL-haloperidol (n=12); NAB-B- nabilone, trial B (n=24); LOS-losartan (n=12); OXY-A- oxytocin, trial A (n=24 at each of three dosage levels); MOR-morphine (n=24); VEH-pooled vehicle data from all 0.75 mV trials (n=332); SPIRO-spironolactone (n=12); MIDZ-midazolam (n=24); PROP-propranolol (n=12); SCOP-scopolamine (n=12); NAB-A- nabilone, trial A (n=24); MIF-A-mifepristone, trial A (n=12); MIF-B-mifepristone, trial B (n=12); VEH-pooled vehicle data from all 1.5 mV trials (n=67) OND-ondansetron (n=24). The number following each drug name indicates parenteral dosage in mg/kg. Figure 2.1.1.4.2 presents effect sizes (Glass' Δ) from Figure 2.1.1.3.1. These are calculated as the change score for each drug trial minus the change score for the pooled vehicle trials, divided by the SD of the latter. The vertical lines indicate the cut-off for statistical significance at $p<0.05$.

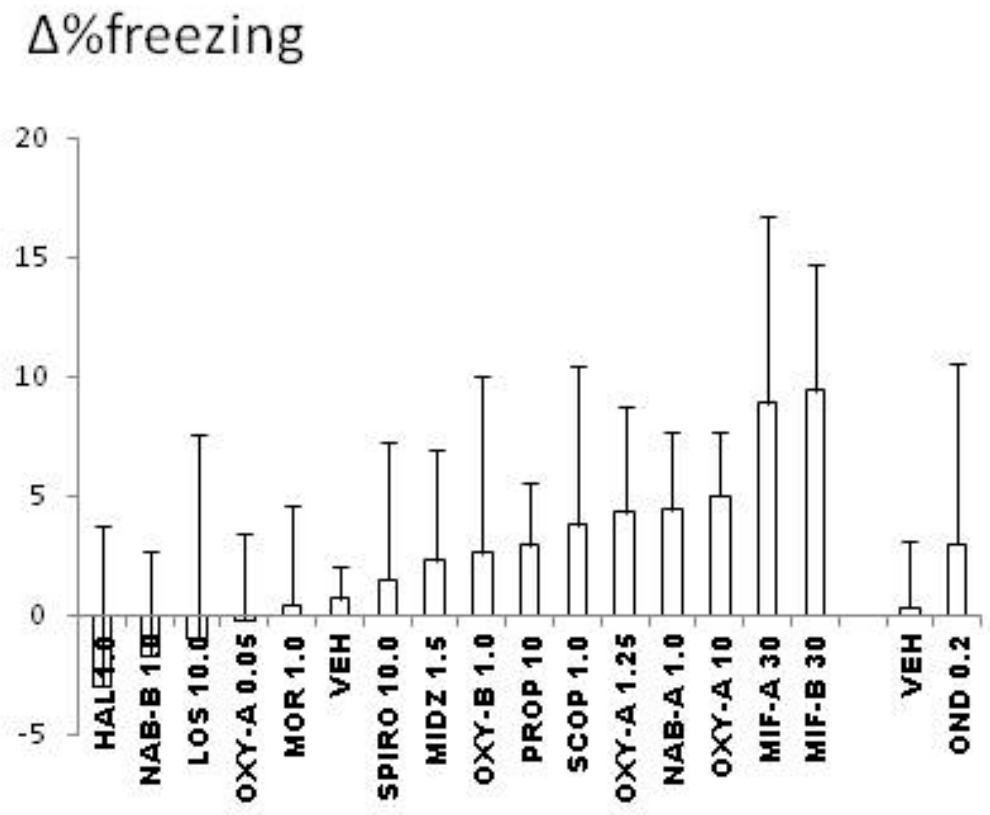

Figure 2.1.1.4.1. Decreases in PR-LTM (percent freezing with various additional drugs.

As may be seen from these two figures, highly significant results were obtained for each of the two mifepristone trials. Significant results were also obtained for the first oxytocin trial (A) at dosages of 1.25 mg/kg and 10 mg/kg, but not at a dosage of 0.05 mg/kg (presented in detail in §2.1.1.2 above). Unfortunately, a second oxytocin trial (B) at 1.0 mg/kg did not replicate these results. Similarly, significant results were obtained for a first nabilone trial (A) at 10 mg/kg (presented in detail in §2.1.1.3 above). However, a second nabilone trial (B) at the same dosage yielded totally negative results, although in this second trial, mifepristone 30 mg/kg was administered concomitantly with the nabilone. No other drug showed significant evidence of reconsolidation blockade.

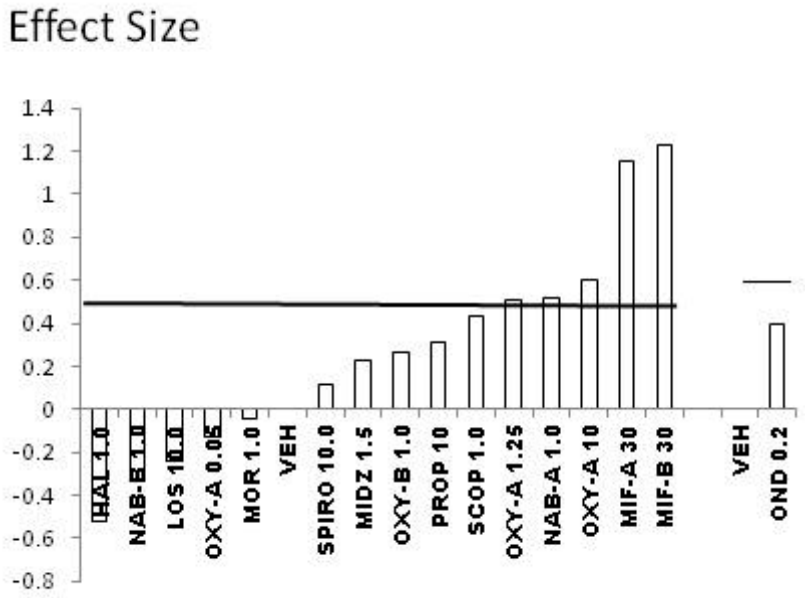

Figure 2.1.1.4.2. Effect sizes for decreases in PR-LTM (precentage of freezing with various additional drugs.

2.1.2. McGill University. We studied clonidine as a potential reconsolidation blocker.

2.1.2.1. Introduction. Clonidine is thought to act at the pre-synaptic level by activating the α_2-autoreceptor, which leads to inhibition of voltage-gated calcium channels and inhibition of norepinephrine release. Clonidine has been found to produce memory impairments in step-down, shuttle box, and passive avoidance tasks. However, the use of clonidine to block memory reconsolidation has yet to be investigated. We examined the use of clonidine as a potential novel treatment for PTSD by testing its effects on the reconsolidation of a conditioned fear memory in rats. We investigated key parameters necessary to develop clinical studies involving reconsolidation blockade with clonidine. We determined the most effective dose through a dose-response curve, established the optimal number of treatments and verified that the observed effects were reconsolidation-specific.

2.1.2.2 Methods. These are described in detail in an attached publication with 5 figures and 46 references (Gamache et al, 2012).[12] In brief, clonidine in doses of 0 (vehicle) 50, 100 or

200 µg/kg was administered intraperitoneally (i.p.). Procedures were the same as in the mifepristone and oxytocin studies described above, with some variations and additions. *Experiment 1* involved the measurement of PR-LTM as previously described. *Experiment 2* involved the measurement of NR-LTM as previously described. *Experiment 3* involved the measurement of PR-STM as previously described. In *Experiment 4,* rats underwent the same procedure as in experiment 1 but received only 0 or 200 µg/kg of clonidine. Then after the test on Day 10, rats were allowed two days of rest before undergoing habituation, new conditioning and testing in a different experimental chamber with a different scent, in order to change the context. Rats were then newly conditioned using a tone of a different frequency in order to measure conditionability. In *Experiment 5* rats were habituated, trained and reactivated as in experiment 1. However, they then underwent reactivation on Day 2, 3 and 4, each time followed by an injection of 0 or 100 µg/kg clonidine. PR-LTM was then tested on Days 5 and 12.

2.1.2.3. Results. These are also described in detail in the same attached publication.[12] The following summarizes the most important results. For all experiments, no significant gender main effect or interaction were observed for freezing to the tone. A repeated-measures ANOVA across days revealed no difference in freezing scores between males and females for any experiment. This lack of gender differences allowed us to combine the freezing scores for males and females for each experiment.

2.1.2.3.1. Experiment 1. Clonidine was effective in reducing PR-LTM at all tested doses, and its effect was long-lasting as the memory impairment was still observed a week after the treatment. Moreover, clonidine disrupted fear memory reconsolidation in a dose-dependent manner. Clonidine reached its maximum effect at 100 µg/kg; increasing the dose further did not lead to a greater impairment of the conditioned response.

2.1.2.3.2. Experiment 2. NR-LTM was preserved. In other words, no significant reducing effect of clonidine on LTM was observed in the absence of reactivation.

2.1.2.3.3. Experiment 3. Results revealed a similar CR for the clonidine-treated rats compared to the vehicle group at PR-STM but showed a significant decrease in freezing for the clonidine group at PR-LTM, as compared to PR-STM, and as compared to vehicle controls at PR-LTM. These results confirm that post-retrieval clonidine selectivity disrupts reconsolidation of long-term but not short-term memories.

2.1.2.3.4. Experiment 4. To evaluate whether post-reactivation clonidine induces permanent learning impairments, following testing of PR-LTM, we conditioned animals to fear a different tone using a different auditory fear protocol. The highest dose of clonidine (200 µg/kg) or vehicle was chosen for this experiment to ensure that if no impairments were observed, it could not be attributed to the use of a low concentration. Both the clonidine and vehicle groups exhibited similar CR levels on the two test days, indicating that administering clonidine after reactivation does not induce a long-lasting, generalized fear learning impairment.

2.1.2.3.5. Experiment 5. To assess whether a greater memory impairment could be achieved, we trained animals as in Experiment 1, except that we reactivated them 3 times over 3 days. Following each reactivation session, rats received an injection of clonidine 100 µg/kg or vehicle. We found a significant decrease in the CR for the clonidine-treated group only between reactivations 1 and 2, and reactivations 2 and 3. The results indicated that reduction of PR-LTM by clonidine was effective after one treatment and reached its maximum effect after 2 treatments.

2.1.2.4. Comment. The results of this study demonstrate the effectiveness of clonidine in persistently impairing fear memory retention through reconsolidation blockade in rats. All tested doses of clonidine showed effectiveness in reducing post-reactivation fear memory retention in a long-lasting and dose-dependent manner. The dose of 100 µg/kg was determined to be optimally effective because it resulted in a greater memory impairment from reactivation to the PR-LTM test than did the 50 µg/kg dose. However, the dose of 200 µg/kg did not induce a larger reduction in freezing than the 100 µk/kg dose, which suggests that the dose-response curve reaches a plateau, and increasing the dose further will not lead to a more substantial decrease in conditioned responding. On the other hand, we did find that the fear memory could be disrupted further with repeated treatments. We established that two reactivation sessions followed by a 100 µg/kg clonidine administration were sufficient to induce maximal memory disruption.

In order to confirm whether reconsolidation blockade is the mechanism underlying the reduction of PR-LTM, we examined key elements that define the reconsolidation process. First, our results demonstrated that the effect of clonidine is selective to the reactivated memory, as no memory impairment was observed when clonidine was administered without prior reactivation. Furthermore, when animals were tested a week after treatment, we did not observe any spontaneous recovery of the conditioned response. Spontaneous recovery is a phenomenon found with extinguished memories, but not after reconsolidation blockade. As reconsolidation is a time-dependent process which is known to affect long-term, but not short-term memory, we also tested the animals 4 hours after reactivation. The results revealed an intact conditioned response at that time point, but impaired PR-LTM the next day.

2.1.3. McLean Hospital

2.1.3.1. Introduction. In this work, we moved beyond behavioral testing of reconsolidation blockade to investigate its underlying synaptic mechanisms. We asked whether reconsolidation blockade affects synaptic plasticity induced by learning, and, if so, how such modifications of synaptic mechanisms in the circuits for a learned behavior might be mediated. We tested the hypothesis that synaptic enhancements in projections from cortex or thalamus to lateral nucleus of the amygdala induced by fear learning are reversed by reconsolidation blockade. We employed the drug rapamycin (Serolimus), which had previously been shown to induce reconsolidation blockade,[13] and which also is approved for human use. The mammalian target of rapamycin (mTOR) kinase regulates protein synthesis at the translational level and has been shown to be critical for fear memory reconsolidation. Rapamycin is an efficient blocker of mTOR kinase. We tested whether blockade of reconsolidation via rapamycin would reverse the learning-induced enhancements in synaptic efficacy in thalamo-lateral amygdala (LA) and cortico-LA projections.

2.1.3.2. Methods. These are described in detail in an attached publication with five figures and 39 references (Li et al, 2013).[14] Briefly, we employed the PR-LTM procedure that has been described above in order to address reconsolidation, but only through Day 3. One hour following Day 3 testing of PR-LTM, the animals were sacrificed in order to perform whole-cell patch-clamp recordings from visualized neurons in slices of the amygdala. In addition, in order to address consolidation only (i.e., in the absence of reconsolidation blockade), and to compare its mechanisms with those underlying reconsolidation, we performed the Day 2 reactivation procedure in the absence of rapamycin (in separate groups of rats). In the prepared slices, we recorded glutamatergic excitatory postsynaptic currents (EPSCs) evoked in LA neurons under voltage-clamp conditions with stimulating electrodes placed to activate either thalamic input (internal capsule) or cortical input (external capsule) to the LA. These two projections deliver the auditory CS information to the LA during fear conditioning. Memory reactivation entailed

presentation of a single CS 24 h post-conditioning, after which rats received an injection of either rapamycin (20 mg/kg, i.p.) or vehicle. We addressed the locus of putative synaptic weakening following reconsolidation blockade. Efficacy of synaptic transmission is determined by a) the probability of neurotransmitter (glutamate) release (P_r), and/or b) postsynaptic responsiveness to neurotransmitter (e.g., as released from single synaptic vesicles, i.e., "quantal amplitude"), as well as by the number of effective synapses. We therefore estimated P_r and quantal amplitude in both thalamic and cortical inputs to the LA following fear conditioning and post-reactivation rapamycin treatment.

2.1.3.3. Results. These are also described in detail in the attached publication.[14] Rapamycin-treated rats showed greater conditioned freezing 24h later (indicative of impaired PR-LTM) compared to their own pre-injection levels, and compared to the vehicle group. When memory reactivation was omitted, increased rapamycin-induced conditioned freezing was not observed. Freezing also did not differ in non-reactivation, rapamycin *vs.* vehicle groups, indicating that impaired PR-LTM was not due to lasting nonspecific effects of rapamycin on fear memory retrieval. The observed decreases in conditioned freezing in rats that received postretrieval rapamycin were associated with a rightward shift in the input-output curves in both thalamic and cortical inputs to the LA compared with vehicle-injected rats, indicating a decrease in the synaptic strength that had previously been enhanced by fear-conditioning. In contrast, synaptic strength remained enhanced in both inputs in rapamycin-injected but non-reactivated rats. Overall, these findings demonstrate the requirement for mTOR activity in maintaining the post-reactivation stability of synaptic potentiation in conditioned fear pathways.

Additionally, we found that the observed increase in synaptic strength in fear-conditioned rats was accompanied by a decrease in the magnitude of paired-pulse ratio (PPR) recorded at a 50-ms interstimulus interval in both studied pathways. Because the magnitude of PPR varies inversely with the basal P_r, the observed increases in synaptic efficacy in the CS pathways of conditioned rats appeared at least in part due to higher pre-synaptic P_r. To estimate post-synaptic responsiveness, we recorded asynchronous single-quanta synaptic events evoked by stimulation of either thalamic or cortical inputs in the external medium where strontium (Sr^{2+}) was substituted for Ca^{2+}. Asynchronous EPSCs may be observed for hundreds of milliseconds following the presynaptic stimulation pulse, thus permitting analysis of quantal responses in specific projections to the target area. Surprisingly, the acquisition of conditioned fear memory did not lead to detectable changes in the amplitude of single-quantum EPSCs in either thalamic or cortical inputs, which suggests a lack of postsynaptic modifications under present conditions.

In contrast to the above, we did not observe PPR changes in rats that had received postretrieval rapamycin *vs.* vehicle. Moreover, postretrieval PPR in rapamycin-treated rats did not differ from that found in the group that did not receive the rapamycin treatment, suggesting that presynaptic enhancements associated with fear conditioning are retained following reconsolidation blockade. PPR in non-reactivated, rapamycin-treated rats was also unaffected. However, single-quantum thalamo-LA and cortico-LA EPSCs were significantly decreased in slices from rats that received postretrieval injections of rapamycin *vs.* vehicle. These results suggest that retention of fear conditioning-produced synaptic enhancements in CS pathways involves the prevention of retrieval-induced decreases in *post*synaptic responsiveness to glutamate. If mTOR signaling-dependent reconsolidation is blocked, synaptic strength returns to the default (pre-conditioning) level.

2.1.3.4. Comment. The results of this study suggest that postretrieval reconsolidation entails a form of synaptic plasticity that is distinct from that involved in the consolidation of conditioned fear memory. Specifically, the decreases in synaptic strength we observed following

the disruption of reconsolidation by rapamycin appear due to modifications in *post*synaptic processes, rather than reversal of *pre*synaptic enhancements produced by initial fear learning. In our experiments, a single CS-US pairing was associated with increased P_r in auditory inputs to the LA. Curiously, although postretrieval rapamycin virtually completely reversed the post-conditioning enhancement in thalamo-LA and cortico-LA EPSCs produced by fear conditioning, it produced only a partial reduction in learned freezing. This discrepancy in electrophysiological and behavioral results suggests first, that there are other mechanisms besides synaptic enhancement in CS pathways to LA that underlie fear learning, and second that these other mechanisms do not require mTOR activity for maintaining their stability. This warrants further investigation. Further experiments will also be required to identify other molecular components, both upstream and downstream, implicated in the mTOR-dependent control of fear memory reconsolidation at the synaptic level.

General comment on the animal work accomplished. Regardless of underlying mechanisms, the animal findings presented above suggest that drugs such as mifepristone, oxytocin, nabilone, clonidine, and rapamycin, which are all approved by human use, could potentially be translated into a novel PTSD treatment procedure based upon pharmacologic reconsolidation blockade.

2.2. Human work

2.2.1 MGH and Dallas VA. In this work, we attempted to translate the above rat findings with mifepristone into psychophysiological work with human subjects. We performed two studies that investigated whether mifepristone given shortly prior to traumatic memory retrieval can reduce psychophysiological responding during subsequent traumatic imagery in subjects with chronic PTSD. (Unlike in the rat work, in which mifepristone was administered *post*reactivation, this work was performed using *pre*reactivation mifepristone, in order to ensure that an adequate level the drug, which had to be administered orally in humans, was achieved at the time of traumatic memory reactivation.) The second study employed d-cycloserine (DCS) in conjunction with mifepristone.

2.2.1.1. First psychophysiological mifepristone study.

2.2.1.1.1. Introduction. We hypothesized that individuals with PTSD whose traumatic memories putatively underwent reactivation during traumatic script preparation (see below) accompanied by mifepristone (reactivation, RM) would show smaller physiological responses during script-driven imagery testing (see below) a week later compared to those who received either mifepristone in the absence of the script preparation procedure (non-reactivation, NRM) or double-placebo controls (PP). For this work, an investigational new drug number for the off-label use of mifepristone was obtained from the U.S. Food and Drug Administration.

2.2.1.1.2. Methods. The methods are described in detail under Studies Two and Three of the attached publication (Wood et al, 2015)[15] with three tables (the last two of which refer to the work described herein) and 33 references.[15] In brief, subjects were males and females ages 18 to 73 who met diagnostic criteria for PTSD, either combat- or noncombat-related. After a full explanation of the procedures, which had been approved by the Partners Human Research Committee, VA North Texas Health Care System Institutional Review Board, and the U.S. Army Medical Research and Materiel Command Human Research Protection Office, subjects gave written informed consent. Recordings of heart rate (HR), skin conductance (SC), and electromyogram (EMG) of the left corrugator and left frontalis facial muscles were made. In pre-clinical studies, reconsolidation blockade was found with a dose of 30 mg/kg, which corresponds to approximately 1800 mg in a 60 kg human. On Day 0 and Day 2, we administered 1800 mg oral mifepristone or placebo. Subjects randomized to the NRM group

received mifepristone on Day 0 and placebo on Day 2. Subjects randomized to the RM received placebo on Day 0 and mifepristone on Day 2. Subjects randomized to the PP group received placebo on Day 0 and Day 2. A double-blind 1:1:1 randomization schedule was utilized. The study medication was well-tolerated by all subjects with few reported side effects.

On Day 0 (non-reactivation), subjects randomized to the NRM group received mifepristone, whereas subjects randomized to the RM or PP group received placebo. Ninety minutes later, all subjects viewed a 90-minute emotionally neutral movie. By design, subjects were not permitted to discuss their combat events or PTSD symptoms on Day 0 to reduce the chances of inadvertent traumatic memory reactivation. On Day 2 (reactivation), subjects randomized to the RM group received mifepristone, whereas subjects randomized to the NRM or PP group received placebo. Ninety minutes later all subjects underwent a script-preparation session, which served to reactivation the memory of the traumatic event that led to the subject's PTSD. Subjects recalled and provided written detail of two traumatic personal experiences, or two aspects of the same traumatic experience, as well as three other personal (non-traumatic) life events. They then selected bodily responses that accompanied each experience. An investigator later composed approximately 30-second scripts portraying each experience and incorporating up to five of the selected bodily responses. Subjects completed a baseline Impact of Event Scale-Revised (IES-R) measuring PTSD symptoms related to each of their five personal events (separately). A psychologist administered the Clinician-Administered PTSD Scale (CAPS) to verify the presence of current PTSD, and the Structured Clinical Interview for DSM-IV (SCID) to evaluate the presence of any other Axis I comorbidity. (In order to reduce the chances of traumatic memory reactivation on Day 0, the CAPS and SCID were administered on Day 2.) On Day 8 (i.e., approximately one week later), subjects underwent the script-driven imagery testing session while physiological measures were obtained. The subject then listened to eleven scripts presented sequentially in pseudorandom order, consisting of the five personal scripts prepared on Day 2 and six standard scripts. Each script presentation consisted of four sequential 30-second periods: baseline, listening, imagery, and recovery. Following the script-driven imagery procedure, subjects completed IES-R scores for each of the five personal events that they had described on Day 2. Response scores for each physiological measure for each script were calculated by subtracting the 30-second baseline period mean from the 30-second imagery period mean. Responses to the two traumatic scripts were averaged and square-root transformed prior to analysis. An *a priori* discriminant function derived from the HR, SC, and lateral frontalis EMG responses during personal traumatic imagery of reference samples of previously studied individuals with and without PTSD using the same technique was used to calculate each subject's posterior probability (PPrb) of being classified with PTSD. The PPrb served as a composite univariate measure of overall physiological responding during script-driven traumatic imagery, eliminating the need for multivariate analyses of physiological responses in the small samples studied. IES-R change scores were calculated for each script by subtracting the Day 2 IES-R score from the Day 8 IES-R score. Change scores for the two traumatic combat scripts were averaged. Single-factor analyses of variance (ANOVAs), with the Group factor having three levels: RM, NRM, PP, were performed for all outcome measures.

2.2.1.1.3. Results. The results are also described in detail in the attached publication.[15] In brief, there were three subject groups: RM n=13, NRM n=15, and PP n=15. When gender was added as a factor to the ANOVA, there were no significant group or gender main effects, or group x gender interaction for physiological PTSD probability score or IES-R change score, so the analyses were collapsed across gender. Results revealed no significant group differences in Day 8 PPrb or IES-R change scores. However, confidence limits in the predicted direction were large enough so that failure to find the hypothesized effect of reactivation mifepristone might have represented a Type II error.

2.2.1.1.4. Comment. The results of this study failed to show significant differences among reactivation mifepristone, non-reactivation mifepristone, and placebo subjects. The dose of mifepristone given, 1800 mg, is greater than four times the dose shown to induce inhibition of GR receptors (Bertagna et al, 1984), suggesting that the lack of effect shown was not due to a low dosage. These negative findings are further discussed under General Comments below.

2.2.1.2. Second psychophysiological mifepristone study

2.2.1.2.1 Introduction. Successful pharmacological blockade of memory reconsolidation depends upon two steps. First the memory must be destabilized by its reactivation (recall). Second, the drug must interfere with the reconsolidation of the reactivated memory. The absence of a reconsolidation blockade effect in the first study above may have resulted from failure to destabilize the memory in the first place, rather than inadequacy of the reconsolidation blocker. Results of a study in animals that had been trained under highly stressful conditions suggest that memory traces formed under such conditions resist destabilization and thus are inaccessible to reconsolidation blockers.[16] However, in that study when the administration of the reconsolidation blocker (midazolam) was preceded by pre-reactivation d-cycloserine (DCS), reconsolidation blockade became successful, suggesting that DCS may promote the destabilization of resistant memory traces. DCS acts as a partial agonist at brain N-methyl-D-aspartate (NMDA) receptors, which have been implicated in memory destabilization in animals. The traumatic memories of individuals with PTSD have by definition been formed under highly stressful conditions and thus may be particularly resistant to destabilization. We hypothesized that individuals with PTSD who underwent memory reactivation via script preparation accompanied by reactivation mifepristone plus pre-reactivation DCS (RMD) would show smaller physiological responses during script-driven imagery testing a week later compared to those who received two placebos (PL).

2.2.1.2.2. Methods. Methods for this study are also described in detail in the attached publication.[15] Briefly, subjects were males and females ages 18 to 62 who met diagnostic criteria for PTSD (combat- and noncombat-related). After a full explanation of the procedures, which had been approved by the Partners Human Research Committee, VA North Texas Health Care System Institutional Review Board, and the U.S. Army Medical Research and Materiel Command Human Research Protection Office, subjects gave written informed consent. The use of DCS was approved by the FDA under the same IND number as in the first study. DCS reaches peak blood levels 4 to 8 hours after oral administration. Therefore, we administered either 100 mg oral DCS plus 1800 mg oral mifepristone, or two placebos. A double-blind 1:1 randomization schedule, stratified by gender, was utilized. The study medication was well-tolerated with few reported side effects. Physiological measures were obtained as in the first study.

On Day 0, a psychologist administered the CAPS and SCID. On Day 7, subjects randomized to the RMD group received DCS approximately 4 hours prior to mifepristone administration. Mifepristone was administered 90 minutes prior to memory retrieval (i.e., script preparation). Subjects randomized to the PL group received matching placebo capsules at each time point. All subjects then underwent a script preparation session as described in the first study. Subjects also completed IES-R scores. On Day 14, subjects underwent the script-driven imagery session as in the first study. Between-group (RMD vs. PL) Student's t-tests were performed for all outcome measures. Two-way ANOVA was performed to incorporate gender into the analyses.

2.2.1.2.3. Results. Group sizes were: RMD *n*=16, PL *n*=15. A two-way ANOVA yielded a main effect of gender on physiological PTSD probability score, $F(1,27)=5.25$ ($p=0.03$), with females showing higher levels of overall reactivity. However there was no significant main group x gender interaction, so the analyses were collapsed across Gender. The group difference in Day 14 physiological PTSD probability score was not significant. There were also no significant group differences on any individual physiological response measure. Nor was there a significant group difference in IES-R change scores. Again, confidence limits in the predicted direction were large enough so that failure to find the hypothesized effect of reactivation mifepristone might have represented a Type II error.

2.2.1.2.4. Comment. The results of the second psychophysiological study also revealed no significant difference between the group receiving mifepristone plus DCS and the placebo control group. We had hoped to enable mifepristone-induced reconsolidation blockade by promoting traumatic memory destabilization with DCS, but according to the present results, this was not achieved.

General Comment on the human psychophysiological work accomplished. Disappointingly, the results of the above two studies failed to show significant effects of mifepristone, with or without d-cycloserine administered prior to traumatic memory retrieval, on subsequent physiological responses during script-driven traumatic imagery, or on change in PTSD symptoms assessed by the Impact of Events Scale (IES-R). However confidence interval analyses indicated that we cannot entirely rule out the possibility of Type II error having played a role in the negative results. Failure to find significant differences between groups in these studies may reflect an insensitivity of the outcome measures. Although heart rate, skin conductance, and electromyogram responses have been found able to identify individuals with versus without PTSD during script-driven traumatic imagery, their sensitivity is only fair.[17] They may not always be able to detect changes induced by a single dose of medication. The two studies also failed to find pharmacological effects on self-reported PTSD symptoms quantified by the IES-R. However, it may be unrealistic to expect a therapeutic effect of a single session of memory reactivation plus drug.

Another possible explanation for the negative results could be a floor effect. The PPrb scores in the control groups in the two studies ranged from only 0.40 to only 0.44, meaning that the average PTSD control subject had less than a 50% likelihood of being psychophysiologically classified as having PTSD. These PPrb scores are substantially lower than we have previously seen in persons with PTSD. The CAPS ranged from 57 to 67, which is consistent with only mild to moderate PTSD. Hence our subjects may not have had severe enough PTSD for us to be able to detect an effect of the drug interventions. The recruitment of quality research subjects is an ongoing difficulty faced in clinical research. Individuals recruited from the community, with the incentive of a participation fee, differ from a treatment-seeking population. Persons with the most severe PTSD may be hesitant to volunteer for research studies.

The tests of the two studies' hypotheses consisted of cross-sectional comparisons of physiological reactivity between subject groups. Baseline physiological reactivity was not assessed in these studies out of a fear of habituating the subject to the script-driven imagery procedure. However, it is possible that a repeated-measures design that measured changes in physiological reactivity both before and after the interventions could have been more sensitive to the hypothesized effects. Additionally, we did not obtain physiological measures during the preparation of the traumatic scripts. Such data might have provided a validity check on the strength of memory retrieval at the time, and the resulting degree of putative memory destabilization. These design modifications should be considered in future studies. Baseline

physiological testing might also be used to select those individuals who show heightened reactivity, so as to avoid potential floor effects and target those individuals with more severe PTSD.

It is also possible that the script-driven imagery procedure is sometimes insufficient to induce traumatic memory destabilization, even when it is preceded by the administration of DCS. The choice to give the candidate reconsolidation blockers before memory retrieval was dictated by the consideration that oral propranolol and mifepristone take approximately 90 minutes to reach peak plasma levels in the human body. Because reconsolidation begins only a few minutes after memory reactivation, post-reactivation administration of these drugs may produce negative results because there will not be sufficient time for their effect to be exerted before a substantial degree of reconsolidation has already occurred. However, having selected this design, we cannot rule out the possibility that the propranolol or mifepristone given in advance may have attenuated memory reactivation during script preparation and thereby failed to produce destabilization of the traumatic memories.

2.2.2. McGill University/Douglas Mental Health University Institute. In previous open-label studies,[18] we found that propranolol, when given shortly prior to traumatic memory reactivation, decreased PTSD symptoms over several treatment sessions. Here we attempted to replicate and extend these results in a randomized, double-blind, placebo-controlled trial. The results of this study were presented as a poster at the 2013 Society of Biological Annual Meeting and are currently being prepared for publications. A copy of the poster is attached.

2.2.2.1. Methods

2.2.2.1.1. Subjects. Adult men and women aged 18-65 years suffering from DSM-IV-TR chronic PTSD were recruited from an outpatient clinic population or via advertisements in the local media. Exclusion criteria were: (i) Basal systolic blood pressure < 100 mm Hg; (ii) basal heart rate < 55 beats per min.; (iii) medical conditions contraindicating the administration of propranolol, including (but not limited to) heart problems, hypotension, respiratory disorder, kidney disease, thyroid disorder, and diabetes; (iv) current use of medication that involved potentially dangerous interactions with propranolol including (but not limited to) other beta-blockers, anti-arrhythmics, and calcium channel blockers; (v) women who were pregnant or breast feeding; (vi) borderline personality disorder, “complex” PTSD, mild PTSD as determined by a pre-treatment CAPS score below 45 at visit 0 and by a PTSD Check List (PCL) score below 44 at visit 1 (i.e., before randomization), bipolar disorder, psychosis, current substance or alcohol dependence, active suicidal ideation; (vii) a score below 4 (i.e., below moderately ill) on the severity scale of the Clinical Global Impression scale. (viii) current participation in psychotherapy other than supportive; (ix) involved in litigation; (x) strong dissociative tendencies, as evidenced by a mean score > 20 on the Dissociative Experience Scale; and (xi) suspected or confirmed traumatic brain injury. Note: Individuals taking SSRIs or serotonin–norepinephrine (SNRIs) reuptake inhibitors were not excluded so long as the treatment regimen had not been changed within the month prior to the study. Rather, they were asked to slightly delay their medication dose on the day they received the study treatment.

Thirty Ss (20F, 10M) randomized to propranolol (PROP) presented for the first treatment session; mean age=36.2 (SD=9.6); mean education=14.6 (SD=3.1); 21 completed treatment and underwent the post-treatment assessment. Twenty-three Ss (12F, 11M, group difference p=0.40) randomized to placebo (PLA) presented for the first

treatment session; mean age=43.7 (SD=11.0), p=0.01; mean education=15.3 (SD=3.2), p=ns; 20 completed treatment and underwent the post-treatment assessment.

2.2.2.1.2. Instruments included the PTSD Checklist-Specific Version (PCL) and the Clinician-Administered PTSD Scale (CAPS). The PCL was administered prior to each treatment session and at the post-treatment assessment with reference to the preceding week. The CAPS was administered at one-week pre- and post-treatment.

2.2.2.1.3. Procedure. At the pre-treatment assessment, a one-page "script" of the S's event that caused the PTSD was prepared. A week later, there began six weekly treatment sessions. At each session, the S received 1 mg/Kg short-acting oral PROP or PLA (same for each session), waited 60-min., read the script aloud to an investigator and then engaged in mental imagery of the personal traumatic event the script portrayed for 10-min.

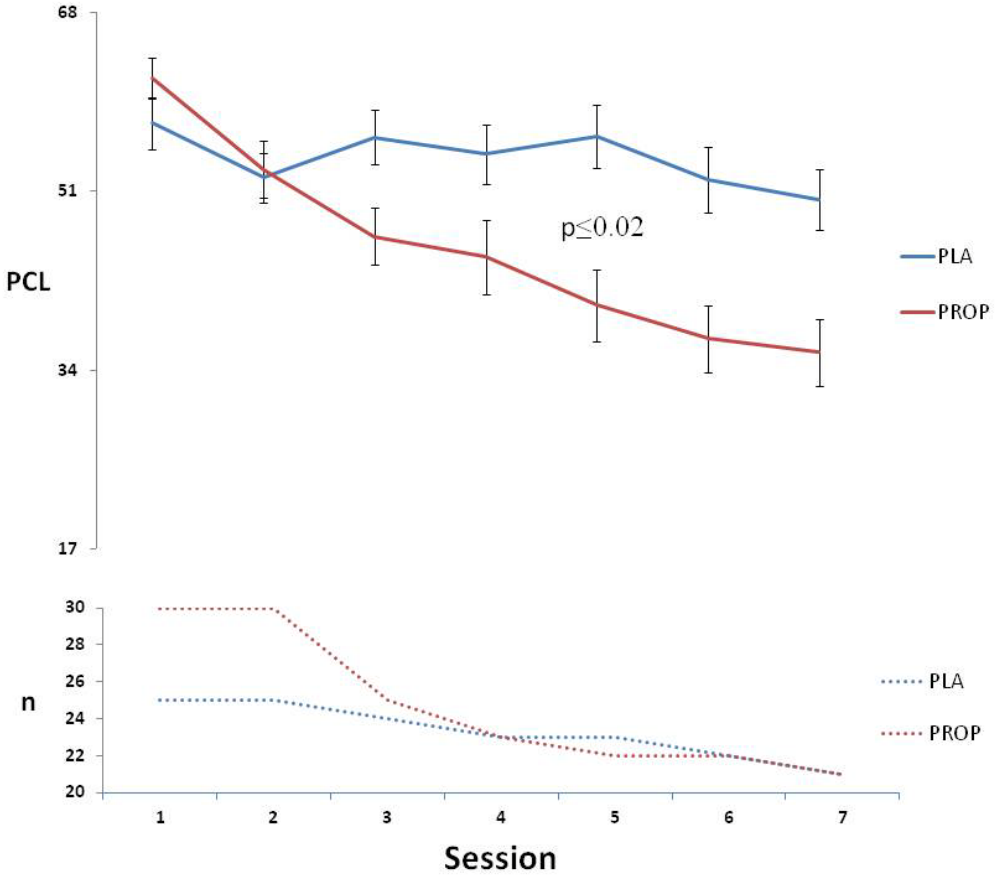

Figure 2.2.2.2.1. Top: Weekly PCL scores. Bottom: Number of subjects who completed the PCL.

2.2.2.2. Results

2.2.2.2.1. PTSD Checkist. Weekly PCL scores are shown in Figure 2.2.2.2.1 Beginning with treatment Session 3, and continuing until the post-treatment assessment (designated Session 7), PCL scores were significantly lower (p≤0.02) in subjects who were receiving weekly propranolol than in subjects who were receiving placebo.

2.2.2.2.2. Clinician-Adminstered PTSD Scale. Pre-and post-treatment CAPS scores in the 21 PROP and 20 PLA Ss who completed all six treatment sessions appear in Figure 2.2.2.2.2. Change scores were subjected to two-factor (Gender, Drug) analysis of covariance (ANCOVA) with age as a covariate. Neither the Gender main effect nor the Gender x Drug interaction was statistically significant. The Drug main effect yielded $F(1,37)=3.4$, $p=0.04$. Collapsed across Gender, within-group pre- to post-treatment effect sizes, calculated as decrease in CAPS scores divided by pre-treatment standard deviation, were as follows: propranolol group 1.6, placebo group 0.7.

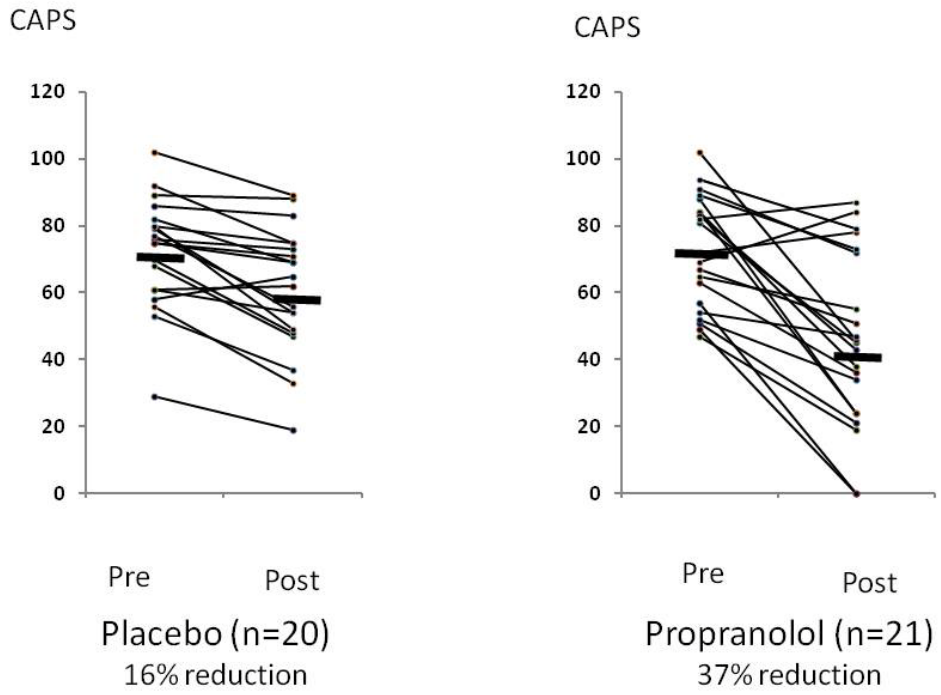

Figure 2.2.2.2.2. Pre- and post-treatment CAPS scores for completers. Heavy bars indicate means.

2.2.2.2.3. Intent-to-treat (ITT) analyses on CAPS scores. A sensitivity analysis was performed that assigned to each subject with a missing post-treatment CAPS score the mean score of the *placebo* group. Applying the same ANCOVA to these data, the Drug main effect remained significant: $F(1,49)=2.8$, $p<0.05$.

2.2.2.2.4. Follow-up. Too few subjects returned for a scheduled 6-month assessment to permit data analysis.

2.2.2.3. Comment. These results indicate that a series of weekly, brief, imaginal exposures to the traumatic event were more effective in reducing PTSD symptoms when these exposures were preceded by propranolol than by placebo. The within-group effect size for reduction in total CAPS score of 1.6 compares favorably with the effect sizes reported for the current treatment of choice for PTSD, viz., cognitive behavior therapy (CBT). Yet the duration of

imaginal exposure to the traumatic event in the present study was less than one-tenth that required by CBT. It is plausible that the superior therapeutic results achieved in the propranolol group were due to blockade of reconsolidation of the trauma memory that was activated by the imaginal exposure. However, further studies that include appropriate controls will be required to establish this, including administration of drug in the absence of reactivation, measurement of symptoms a few hours following the exposure, and long-term follow-up in an adequate sample to permit the evaluation of spontaneous recovery of PTSD symptoms.

3. KEY RESEARCH ACCOMPLISHMENTS

3.1. a) Replication of an earlier finding that post-reactivation mifepristone blocks the reconsolidation of conditioned fear memories in rats, further supporting the role of glucocorticoids in memory reconsolidation; b) The original discovery that propranolol prevents this effect, supporting the interaction of adrenergic and glucocorticoid influences on memory reconsolidation.

3.2. The original discovery that post-reactivation administration of clonidine impairs reconsolidation of auditory fear memories in rats, further supporting the role of adrenergic activity in memory reconsolidation, and offering another candidate drug for use with memory reactivation in PTSD.

3.3. The original discoveries that a) the mammalian target of rapamycin (mTOR) kinase-dependent signaling mediates stabilization of fear conditioning-produced synaptic strengthening in the conditioned stimulus pathways following memory recall through a post-synaptic mechanism; and that b) rapamycin blocks this effect.

3.4. In the context of a first, double-blind, placebo controlled treatment trial, the original discovery that a series of six treatment sessions with propranolol plus memory reactivation is efficacious in reducing symptoms in chronic PTSD.

4. REPORTABLE OUTCOMES

4.1 Publications (all attached)

Gamache K, Pitman RK, Nader K. Preclinical evaluation of reconsolidation blockade by clonidine as a potential novel treatment for posttraumatic stress Disorder. *Neuropsychopharmacology* 2012;37:2789-2796.

Li Y, Meloni EG, Carlezon WA Jr, Milad MR, Pitman RK, Nader LK, Bolshakova VY. Learning and reconsolidation implicate different synaptic mechanisms. *Proceedings of the National Academy of Science USA* 2013;110:4798-4803

Pitman RK, Milad MR, Igoe SA, Vangel MG, Orr SP, Tsareva A, Gamache K, Nader K. Systemic mifepristone blocks reconsolidation of cue-conditioned fear; propranolol prevents this effect. *Behavioral Neuroscience 2011;125:632-638.*

Wood NE, Rosasco ML, Suris AM, Spring JD, Marin M, Lasko NB, Goetz J, Fischer AM, Orr SP, Pitman RK. Pharmacological blockade of memory reconsolidation in posttraumatic stress disorder: three negative studies. 2015; 225:31-39.

4.2. Poster presentation (attached)

Brunet A, Saumier D, Tremblay J, Orr SP, Pitman RK. Randomized placebo-controlled trial of propranolol plus trauma memory reactivation for PTSD. Presented at the 69th Annual Scientific Meeting of the Society for Biological Psychiatry, New York, NY, May 10, 2014.

5. CONCLUSION

Animal and human studies offer promise for the development of a novel treatment for PTSD based upon pharmacological blockade of memory reconsolidation. We have identified a drug approved for human use that blocks reconsolidation of conditioned fear in rats. We have clarified the post-synaptic mechanism of reconsolidation blockade. We have completed a randomized, controlled, double-blind study showing that a series of six treatment sessions with propranolol plus traumatic memory reactivation is efficacious in reducing symptoms in chronic PTSD. *This represents a new, translational treatment for this disorder.*

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7. PERSONNEL WHO RECEIVED PAY FROM THE RESEARCH EFFORT. The following list includes anyone who received any pay at any time at any of the three sites during the five-year project, including summer interns.)

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8. APPENDICES/SUPPORTING DATA

Four reprints and one poster presentation are attached.

Neuropsychopharmacology (2012), 1–8

www.neuropsychopharmacology.org

Preclinical Evaluation of Reconsolidation Blockade by Clonidine as a Potential Novel Treatment for Posttraumatic Stress Disorder

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Exposure to traumatic events can lead to posttraumatic stress disorder (PTSD). Current PTSD treatments typically only produce partial improvement. Hence, there is a need for preclinical research to identify new candidate drugs and to develop novel therapeutic approaches. Animal studies have indicated that fear memories can be weakened by blocking restabilization after retrieval, a process known as reconsolidation. Furthermore, evidence suggests that there are important alterations of the noradrenergic system in PTSD, and hence it may be of interest to study drugs that target this pathway. Here, we investigated the efficacy of clonidine, an α_2-adrenoreceptor agonist, to block reconsolidation in an animal model of persistent traumatic memories. Using an auditory fear conditioning paradigm in rats, we tested the efficacy of clonidine to weaken fear memory retention when administered systemically after retrieval. We evaluated dosage, number of treatments, and specificity in reconsolidation blockade. We found that postretrieval administration of clonidine disrupts fear-related memories in a dose-dependent manner and that two treatments are sufficient for maximal memory impairment. Furthermore, we determined that this effect is long lasting and specific to reconsolidation processes as shown by the selectivity to affect reactivated memories and the absence of spontaneous recovery and of postreactivation short-term memory impairment. Our results demonstrate the efficacy of systemic administration of clonidine following retrieval to persistently disrupt fear memory retention through reconsolidation blockade. This study provides important preclinical parameters for future therapeutic strategies involving clonidine to block reconsolidation as a novel treatment for PTSD symptoms.
Neuropsychopharmacology advance online publication, 8 August 2012; doi:10.1038/npp.2012.145

Keywords: clonidine; memory; reconsolidation; fear conditioning; α2-adrenoreceptor agonist; posttraumatic stress disorder

INTRODUCTION

In a substantial minority of individuals, experiencing a traumatic event can lead to posttraumatic stress disorder (PTSD). This condition is characterized by several symptoms including irritability, hypervigilance, avoidance behaviors, intrusive memories, and frequent re-experiencing of the traumatic event through nightmares and flashbacks. PTSD affects 10–20% of people who have experienced a traumatic event. It has a lifetime prevalence of 6.8% in the United States (Kessler *et al*, 2005). Current therapeutic strategies include psychotherapy and pharmacological treatments; however, only 60% of patients will be responsive to these treatments (Davidson *et al*, 2006; Onder *et al*, 2006) and only 20–30% will achieve full remission (Berger *et al*, 2009). Consequently, there is a significant need to develop novel pharmacological approaches to reduce symptoms of PTSD.

A proposed therapeutic strategy involves the modification of memory reconsolidation processes. In order for a new memory to be retained, it has to be stabilized through a mechanism referred to as consolidation. When such a memory is retrieved (recalled), it becomes unstable again for a short period of time, at which point it is susceptible to modifications (Nader and Hardt, 2009). The memory is then restabilized (reconsolidated) in its modified state. In PTSD, flashbacks, nightmares, and recollection of intrusive memories allow the traumatic memory trace to be retrieved and then reconsolidated (Charney, 2004). Impairing reconsolidation of such memories may lead to their weakening and may consequently diminish PTSD symptoms.

In animal models, pharmacological interventions exploit the vulnerable state of a memory after recall in order to impair reconsolidation. Even though there is no animal model that recreates PTSD entirely, fear conditioning is known to model the fear that accompanies reminders of the traumatic event (Pitman *et al*, 1999; Siegmund and Wotjak,

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2006). Studies have shown that fear memories can be weakened by blocking the restabilization process with different drugs, such as protein synthesis inhibitors (Nader *et al*, 2000), *N*-methyl-D-aspartate (Ben Mamou *et al*, 2006), or adrenergic receptor antagonists (Przybyslawski *et al*, 1999; Debiec and Ledoux, 2004) and inhibitors of the mammalian target of rapamycin (Blundell *et al*, 2008; Jobim *et al*, 2012). A disadvantage of these pharmacological agents is that most of them are toxic, administered intracranially, and not approved for humans. In order to more easily extrapolate work in animal models to clinical trials, investigated drugs should be safe for human use.

Evidence suggests that among other physiological alterations, there is increased noradrenergic activity in PTSD patients (Southwick *et al*, 1997, 1999; Boehnlein and Kinzie, 2007). Furthermore, it has been proposed that this hyperactivity is associated with hyperarousal and re-experiencing symptoms present in PTSD (Southwick *et al*, 1997; Boehnlein and Kinzie, 2007). Consequently, drugs that specifically target noradrenergic system hyperactivity and are safe for human use may be of clinical interest. One of those candidate drugs is the α_2-adrenoreceptor agonist clonidine. The effect of clonidine on memory has been shown to be mediated through the α_2-adrenoreceptor subtype (Galeotti *et al*, 2004). These receptors are located both pre- and post-synaptically. Clonidine is thought to act mainly at the presynaptic level by activating the α_2-autoreceptor (Southwick *et al*, 1999; Wilens, 2006), which leads to inhibition of voltage-gated calcium channels and inhibition of norepinephrine release (Southwick *et al*, 1999; Gilsbach and Hein, 2011). Clinically, clonidine is used to induce sedation, analgesia, and hypotension (MacMillan *et al*, 1996; Lakhlani *et al*, 1997), as well as in the treatment of attention-deficit/hyperactivity disorder (Wilens, 2006). Additionally, a few open-label studies have shown beneficial effects of clonidine in treating some PTSD symptoms (Kinzie and Leung, 1989; Harmon and Riggs, 1996; Ziegenhorn *et al*, 2009), but none of these studies used clonidine specifically in combination with traumatic memory retrieval. In animal models, the use of clonidine has been found to produce memory impairments in step-down (Genkova-Papasova and Lazarova-Bakurova, 1988; Genkova-Papazova *et al*, 1997), shuttle box (Hawkins and Monti, 1979; Homayoun *et al*, 2003), and passive avoidance tasks (Galeotti *et al*, 2004); however, the use of clonidine to block memory reconsolidation has yet to be investigated.

The present study aims to examine the use of clonidine as a potential novel treatment for PTSD by testing its effects on the reconsolidation of a fear memory in rats. We investigated key parameters necessary to develop clinical studies involving reconsolidation blockade with clonidine. We determined the most effective dose through a dose–response curve, established the optimal number of treatments, and verified that the observed effects were reconsolidation specific.

MATERIALS AND METHODS

Animals

Equal numbers of male and female Sprague-Dawley rats weighing between 250 and 350 g (Harlan Laboratories, Indianapolis, IN) were co-housed with *ad libitum* access to food and water. Rats were maintained on a 12 h light/dark cycle. All experiments were performed during the light (day) phase. All procedures were approved by McGill Animal Care Committee and complied with the Canadian Council for Animal Care guidelines.

Drugs

Clonidine hydrochloride (Sigma-Aldrich, Canada) was dissolved in sterile saline (0.9% NaCl) to the final concentration (50, 100, or 200 μg/kg) and administered intraperitoneally at a volume of 1 ml/kg (Galeotti *et al*, 2004).

Behavioral Procedure

Rats underwent auditory fear conditioning, reactivation, and testing in the same experimental chamber to further resemble, in our animal model, a PTSD-like intrusive memory in which cue and context are usually not easily separated. The conditioning chamber consisted of a brightly lit plexiglass box (25 × 29 × 29 cm) with stainless steel-grid floor that was enclosed within a sound-attenuating box (Coulbourn Instruments, Whitehall, PA).

Experiment 1. Rats were first habituated to the chamber for 5 min on 2 consecutive days. The following day (day 1), rats were conditioned. Conditioning involved 2 min of acclimation to the chamber after which rats received a single pairing of a tone (30 s, 5 kHz, 75 dB) coterminating with a foot shock (1 s, 0.75 mA). Rats remained in the chamber an additional minute before being returned to their home cages. On day 2, the fear memory was reactivated by placing the animals in the experimental chamber and presenting the tone without the shock. Rats were then removed from the context and clonidine (50, 100, or 200 μg/kg) or its vehicle was administered immediately. On days 3 and 10, animals were tested for postreactivation long-term memory (PR-LTM) with the presentation of a single tone.

Experiment 2. Nonreactivated controls were habituated and trained as in experiment 1, but rats did not receive the reactivation and instead remained in the animal colony where they received the clonidine treatment on day 2.

Experiment 3. As a postreactivation short-term memory (PR-STM) control, animals were habituated, trained, reactivated, and given postreactivation clonidine as in experiment 1. They were tested 4 h after the reactivation session on day 2, and again 24 h later.

Experiment 4. Rats underwent the same procedure as in experiment 1 and received clonidine (200 μg/kg) or vehicle following reactivation. After the test on day 10, rats were allowed 2 days of rest before undergoing habituation, new conditioning, and testing in a different experimental chamber. The conditioning chamber consisted of a dimly lit plexiglass and steel box (25 × 29 × 29 cm) with one curved white plastic wall and one black and white striped wall, enclosed within a sound-attenuating box (Med Associates, VT). A smaller steel-grid floor was used in this design and peppermint-scented water was also vaporized

inside the box to create a different scent than before. Rats were first habituated to the chamber for 5 min on 2 consecutive days. The following day (day 14), rats were newly conditioned. After 3 and a half minutes of acclimation to the chamber, rats received a single pairing of a different frequency tone (20 s, 3 kHz, 85 dB) coterminating with a foot shock (1 s, 1.1 mA). Rats remained in the chamber an additional 2 min before being returned to their home cages.

Experiment 5. Rats were habituated, trained, and reactivated as described in experiment 1. However, rats underwent reactivation on days 2, 3, and 4, each time followed by an injection of clonidine (100 μg/kg) or its vehicle. Rats were tested on days 5 and 12 using the same procedure as above.

Behavior was recorded using FreezeView software (Actimetrics). Freezing, defined as immobilization with the exception of respiration (Blanchard and Blanchard, 1969), was the conditioned response taken as a measure of fear memory retention. Scores are presented as the percentage of time spent freezing during the total duration of the tone.

Statistical Analysis

A repeated-measures analysis of variance (ANOVA) followed by Fisher's *post hoc* analysis was used to compare groups across days. Significance was set as $p<0.05$.

RESULTS

For all experiments, no significant sex main effect or interaction was observed for freezing to the tone. A repeated-measures ANOVA across days revealed no difference in freezing scores between males and females for any experiment. This lack of sex differences allowed us to combine the freezing scores for males and females for each experiment.

Pre-tone freezing was also analyzed with a repeated-measures ANOVA across days and no significant main effect of treatment or interaction was observed for any of the experiments. A main effect of sex was observed on pre-tone freezing only for experiments 1 and 3, where there was a lower pre-tone freezing response in the females. In light of these isolated results, the lack of treatment effect on pre-tone freezing, and because our measure of memory retention was tone-related freezing, pre-tone freezing was not further investigated.

Experiment 1: Postreactivation Administration of Clonidine Impairs Reconsolidation of Auditory Fear Memories in a Dose-Dependent Manner

We evaluated whether clonidine is effective at disrupting fear memory reconsolidation when administered systemically at 50, 100, or 200 μg/kg. We conditioned the animals on day 1 and reactivated them the following day by exposing them again to the conditioning chamber and the tone. After reactivation, animals received an injection of clonidine or its vehicle and were tested for memory retention a day later. To establish if the effects of clonidine were long lasting, rats were also tested again on day 10 (Figure 1a). Clonidine was effective at blocking memory reconsolidation at all tested doses, and its effect was long lasting as the memory impairment was still observed a week after the treatment (Figure 1b–d). A repeated-measures ANOVA revealed a main effect of treatment ($F(1,34)=6.08$, $p<0.05$ for 50 μg/kg; $F(1,48)=10.61$, $p<0.01$ for 100 μg/kg; $F(1,37)=7.99$, $p<0.01$ for 200 μg/kg) and day ($F(2,68)=9.09$, $p<0.001$ for 50 μg/kg; $F(2,96)=36.04$, $p<0.001$ for 100 μg/kg; $F(2,74)=22.05$, $p<0.0001$ for 200 μg/kg). A significant treatment × day interaction was observed for 100 μg/kg ($F(2,96)=4.66$, $p<0.05$) and 200 μg/kg ($F(2,74)=5.71$, $p<0.01$). Subsequent Fisher's *post hoc* tests indicated significant differences between the clonidine-treated group and the controls at both memory retention

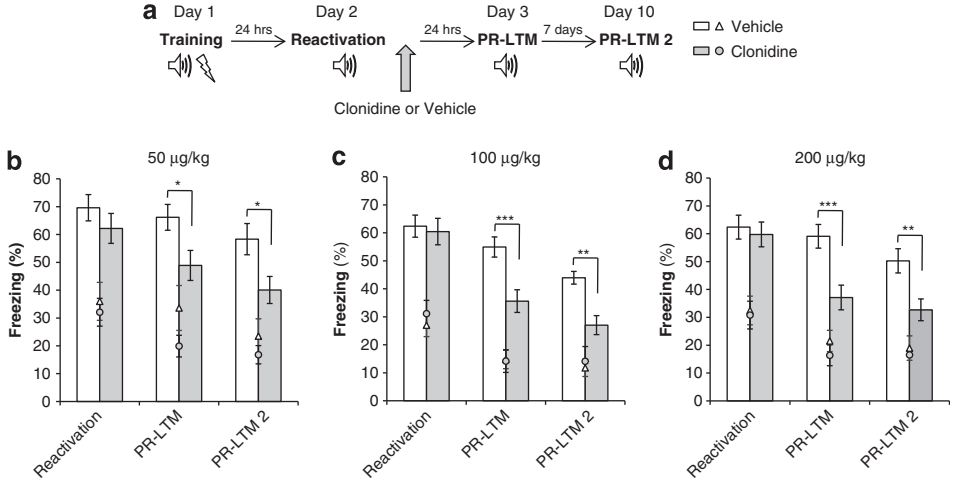

Figure 1 Postreactivation administration of clonidine impairs reconsolidation of auditory fear memories. (a) Schematic of the experimental design. Rats received a single systemic injection of clonidine or its vehicle immediately after a reactivation session, and were tested for postreactivation long-term memory 1 day (PR-LTM) and 1 week later (PR-LTM 2). A dose of (b) 50 μg/kg ($n=20$), (c) 100 μg/kg ($n=25$) and (d) 200 μg/kg ($n=20$) was effective at impairing memory reconsolidation compared with the vehicle group (respectively, $n=16$, $n=25$, and $n=20$) as shown by an impaired conditioned response (freezing) at both time points. Bars represent mean ± SEM freezing to the tone. Markers represent the mean ± SEM freezing before the onset of the tone. Statistical significance: *$p<0.05$, **$p<0.01$, ***$p<0.001$.

tests (for 50 μg/kg, $p<0.05$ for both tests; for 100 μg/kg, $p<0.001$ for PR-LTM and $p<0.01$ for PR-LTM 2; for 200 μg/kg, $p<0.001$ for PR-LTM and $p<0.01$ for PR-LTM 2). In addition, significant freezing decreases were observed within the clonidine group between reactivation and both PR-LTM performances (for 50 μg/kg, $p<0.05$; for 100 μg/kg, $p<0.001$; for 200 μg/kg, $p<0.001$). Taken together, the present data suggest that clonidine disrupted fear memory reconsolidation in a dose-dependent manner. Clonidine reached its maximum effect at 100 μg/kg, as increasing the dose further did not lead to a greater impairment of the conditioned response in the treated group.

Experiment 2: Reconsolidation Blockade by Clonidine Is Selective to Reactivated Fear Memories

We assessed whether the effect of clonidine on reconsolidation was dependent on memory reactivation. We injected clonidine at a dose of 100 μg/kg 24 h after training without exposing the animals to the conditioning chamber and tone. Rats were tested for memory retention on days 3 and 10 (Figure 2a). No significant effect of clonidine (repeated-measures ANOVA, $F(1,22)=0.002$, $p>0.05$) was observed in the absence of reactivation, as compared with the vehicle-injected group 1 day and 1 week after receiving the treatment (Figure 2b). In addition, a repeated-measures ANOVA showed no significant effect of day ($F(1,22)=1.34$, $p>0.05$) and no treatment × day interaction ($F(1,22)=0.39$, $p>0.05$). Thus, clonidine disrupts reconsolidation of an auditory fear memory only when administered following reactivation of that memory.

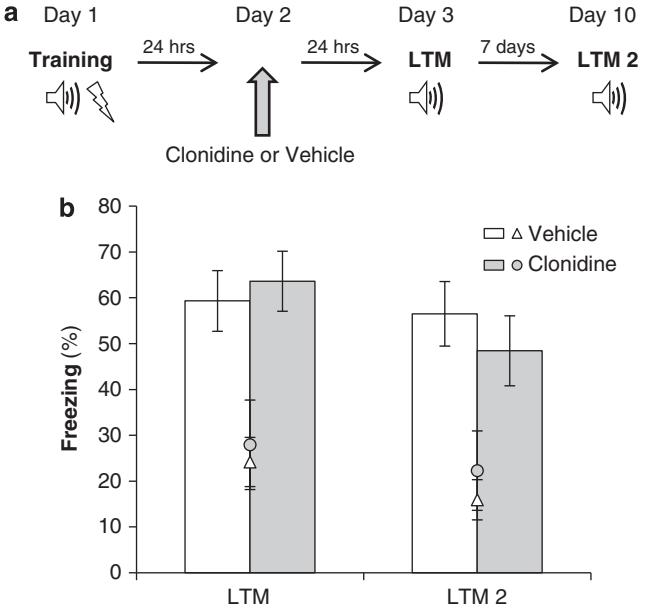

Figure 2 Clonidine does not impair retention of nonreactivated fear memories. (a) Schematic of the experimental design. Rats received a single systemic injection of clonidine (100 μg/kg) or its vehicle without a memory reactivation session and were tested for long-term memory retention 1 day (LTM) and 1 week later (LTM 2). (b) Clonidine-treated rats ($n=12$) showed a similar conditioned response (freezing) to the vehicle group ($n=12$) when tested 24 h or 1 week after injection. Bars represent mean ± SEM freezing to the tone. Markers represent the mean ± SEM freezing before the onset of the tone.

Experiment 3: Postreactivation Administration of Clonidine Does Not Impair Short-Term Fear Memories

To rule out the possibility that nonspecific effects of postreactivation clonidine create temporary dysfunctions of the memory system, we trained and reactivated rats as described before. After reactivation, animals received 100 μg/kg of clonidine or vehicle and were tested for memory retention 4 and 24 h later (Figure 3a). If the memory impairment seen at PR-LTM is due to reconsolidation blockade, then animals should show an intact conditioned response 4 h after reactivation (PR-STM) but reduced freezing behavior 24 h later (PR-LTM). A repeated-measures ANOVA showed a significant main effect of treatment ($F(1,19)=5.49$, $p<0.05$) and day ($F(2,38)=10.9$, $p<0.001$), but no treatment × day interaction ($F(2,38)=2.21$, $p>0.05$; Figure 3b). Nevertheless, Fisher's *post hoc* test revealed a similar conditioned response for the clonidine-treated rats as compared with the vehicle group at PR-STM ($p>0.05$) but showed a significant decrease in freezing for the clonidine group at PR-LTM as compared with PR-STM ($p<0.001$) and to controls at PR-LTM ($p<0.001$). Hence, the results confirm that postretrieval clonidine selectivity disrupts reconsolidation of long-term memories.

Experiment 4: Reconsolidation Blockade by Clonidine Does Not Impair the Ability to Learn New Fear Memories

To evaluate whether postreactivation clonidine could induce permanent learning impairments, we conditioned animals to fear a different tone using a different auditory

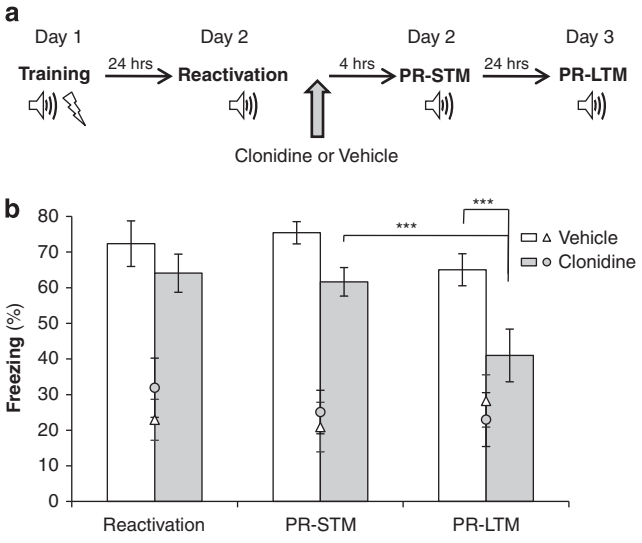

Figure 3 Postreactivation administration of clonidine does not impair short-term fear memories. (a) Schematic of the experimental design. Rats received a single systemic injection of clonidine (100 μg/kg) or its vehicle immediately after a reactivation session and were tested 4 h later for postreactivation short-term memory (PR-STM) and 1 day later for postreactivation long-term memory (PR-LTM). (b) Clonidine-treated rats ($n=12$) showed a similar conditioned response (freezing) to the vehicle group ($n=9$) when tested 4 h after reactivation, but reduced freezing behavior 1 day after injection. Bars represent mean ± SEM freezing to the tone. Markers represent the mean ± SEM freezing before the onset of the tone. Statistical significance: ***$p<0.001$.

fear protocol. After receiving a postreactivation injection of clonidine (200 μg/kg) or vehicle, and a memory retention test 1 and 7 days later, rats were trained again and tested for memory of the new tone (Figure 4a). The highest dose was chosen for this experiment to ensure that if no impairments were observed, it could not be attributed to the use of a low concentration. We hypothesized that if the clonidine-related memory impairment is selective to reconsolidation blockade, then the fear response of the previously treated animals should be similar to the controls when tested for memory of the new tone. A repeated-measures ANOVA revealed no significant main effect of treatment ($F(1, 22) = 0.002$, $p > 0.05$), day ($F(1, 22) = 2.17$, $p > 0.05$), and no treatment × day interaction ($F(1, 22) = 0.7$, $p > 0.05$; Figure 4b). As both groups exhibited similar levels of conditioned response on the two test days, our data indicate that administering clonidine after reactivation does not induce a long-lasting, generalized fear learning impairment.

Experiment 5: Two Postretrieval Treatments of Clonidine Are Sufficient to Induce Maximal Disruption of Fear Memories

To assess whether a greater memory impairment could be achieved using a dose of 100 μg/kg, we trained animals as described before but we reactivated them 3 times over 3 days. Following each reactivation session, rats received an injection of clonidine or its vehicle. Rats were also tested 24 h after the last treatment and 1 week later (Figure 5a). A repeated-measures ANOVA revealed a significant main effect of treatment ($F(4, 116) = 9.91$, $p < 0.01$) and day ($F(4, 116) = 26.04$, $p < 0.001$), and a treatment × day interaction ($F(4, 116) = 2.70$, $p < 0.05$). Fisher's *post hoc* test found a significant decrease in conditioned response for the clonidine-treated group between reactivations 1 and 2 ($F(4, 116) = 26.04$, $p < 0.001$) and reactivations 2 and 3 ($F(4, 116) = 26.04$, $p < 0.05$; Figure 5b). Although the third treatment showed a trend toward additional freezing reduction, it did not have a significant additive effect. The *post hoc* analysis also revealed a significant difference between the treated rats and the controls at days 2, 3, 4, and 12 (all $p < 0.01$). Altogether, the results indicate that reconsolidation blockade by clonidine was effective after one treatment and reached its maximum effect after two treatments.

DISCUSSION

This study demonstrates the effectiveness of clonidine in persistently impairing fear memory retention through reconsolidation blockade in male and female rats. We suggest that the combination of memory reactivation sessions followed by clonidine administration represent a potentially novel therapeutic approach to reduce symptoms in PTSD patients.

Dosage and Number of Treatments

All tested doses of clonidine showed effectiveness in reducing postreactivation fear memory retention in a long-lasting and dose-dependent manner. The dose of 100 μg/kg was determined to be optimally effective because it resulted in a greater memory impairment from reactivation to the PR-LTM test than did the 50 μg/kg dose. However, the dose of 200 μg/kg did not induce a larger reduction in freezing than the 100 μg/kg dose, which suggests that the dose–response curve reaches a plateau, and increasing the dose further will not lead to a more substantial decrease in conditioned responding. On the other hand, we did find that the fear memory could be disrupted further with repeated treatments. Indeed, we established that two reactivation sessions followed by a 100 μg/kg clonidine administration were sufficient to induce maximal memory disruption.

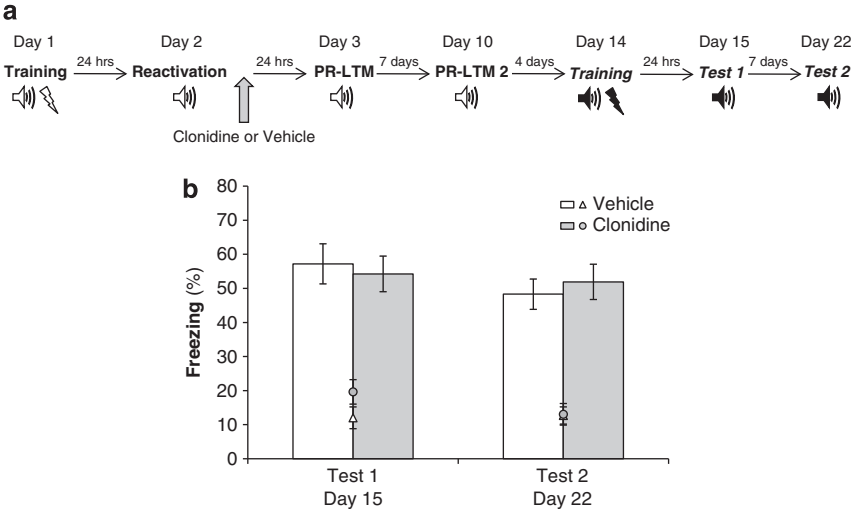

Figure 4 Postreactivation administration of clonidine does not impair the ability to learn new fear memories. (a) Schematic of the experimental design. After receiving a postreactivation injection of clonidine (200 μg/kg) or vehicle, and being tested for memory retention 1 day (PR-LTM) and 1 week later (PR-LTM 2), rats were conditioned to fear a different tone using a different auditory fear protocol. (b) Rats that previously received clonidine ($n = 12$) showed intact fear behavior (freezing) compared with the vehicle-treated animals ($n = 12$) when tested 1 day (test 1) or 1 week later (test 2). Bars represent mean ± SEM freezing to the tone. Markers represent the mean ± SEM freezing before the onset of the tone.

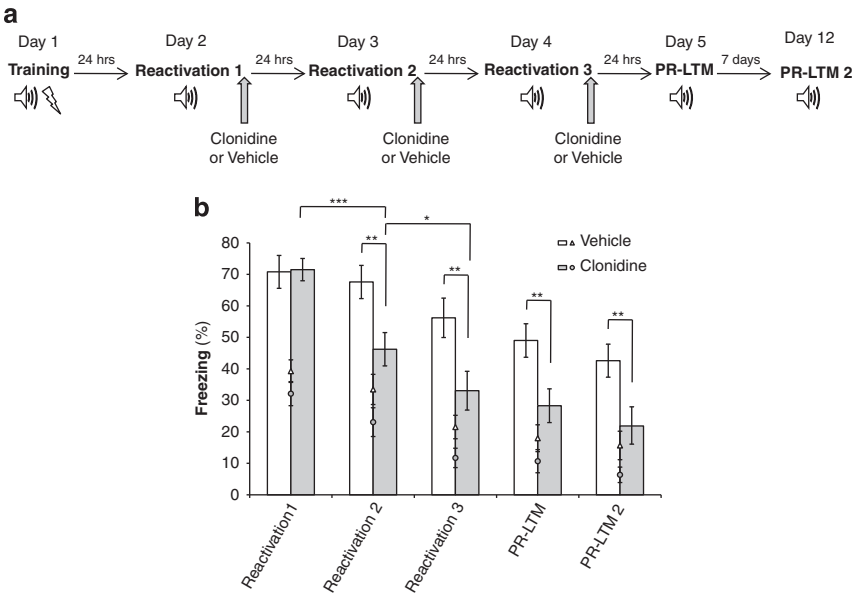

Figure 5 Two postreactivation clonidine treatments are sufficient to maximally impair fear memory retention. (a) Schematic of the experimental design. Rats received a systemic injection of clonidine (100 μg/kg) or its vehicle immediately after a reactivation session for 3 consecutive days and were tested for postreactivation long-term memory 1 day (PR-LTM) and 1 week later (PR-LTM 2). (b) Clonidine-treated rats ($n = 16$) showed an impaired conditioned response (freezing) as compared with the vehicle group ($n = 15$) at each test session. Memory disruption was observed after the first clonidine treatment and reached its maximum after two treatments at day 3. Bars represent mean ± SEM freezing to the tone. Markers represent the mean ± SEM freezing before the onset of the tone. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Our results are consistent with studies showing that clonidine has detrimental effects on memory. In animals, clonidine has been found to produce memory impairments in several learning paradigms ranging from shuttle box (Hawkins and Monti, 1979; Homayoun *et al*, 2003) to avoidance tasks (Galeotti *et al*, 2004; Genkova-Papasova and Lazarova-Bakurova, 1988; Genkova-Papazova *et al*, 1997) and to cue detection (Smith and Aston-Jones, 2011; Brown *et al*, 2012). Some studies in humans have also reported memory impairments associated with clonidine administration in healthy subjects (Riekkinen *et al*, 1999; Hall *et al*, 2001) and in Alzheimer's disease patients (Jakala *et al*, 1999a, b).

It is well known that α_2-adrenoreceptor agonists can induce sedation (Lakhlani *et al*, 1997; MacDonald *et al*, 1997). However, the possibility that a sedative effect of clonidine influenced the behavioral results in our study can be ruled out as we tested the animals 24 h and again 7 days after injection, the time points well beyond the 30–120 min half-life of clonidine in rats (Conway and Jarrott, 1982).

Reconsolidation Specificity

We have shown that postretrieval administration of clonidine is effective in reducing fear-related memory retention. In order to confirm whether reconsolidation is the mechanism underlying the effect, we examined key elements that define the reconsolidation process. First, our results demonstrate that the effect of clonidine is selective to the reactivated memory, as no memory impairment was observed when clonidine was administered without prior reactivation. Furthermore, when animals were tested a week after treatment, we did not observe any spontaneous recovery of the conditioned response. Spontaneous recovery is a phenomenon found with extinguished memories, but not after reconsolidation blockade (Duvarci and Nader, 2004). As reconsolidation is a time-dependent process that is known to affect long-term but not short-term memory (Nader *et al*, 2000; Nader and Hardt, 2009), we also tested the animals 4 h after reactivation. The results revealed an intact conditioned response at that time point but impaired behavior the next day. This demonstrates that clonidine affects postreactivation long-term memory, but not short-term memory. Given that this test was performed only 4 h after clonidine administration, one could argue that the sedative effects of clonidine altered the results at this shorter interval after drug administration. However, the treated rats displayed low levels of freezing during the pre-tone period, indicating an ability to move; thus, the intact freezing levels observed at PR-STM after clonidine administration are unlikely to be attributable to motor impairments due to sedation in these animals. In addition, it is reasonable to believe that the drug was no longer present in the rats' systems at the time of testing because clonidine has a short half-life (30–120 min; Conway and Jarrott, 1982).

Evaluation of the above-mentioned criteria all rule in favor of the implication of reconsolidation processes in the present study. Our results are consistent with several studies investigating reconsolidation blockers either systemically (Debiec and Ledoux, 2004; Blundell *et al*, 2008; Taubenfeld *et al*, 2009; Pitman *et al*, 2011) or intracranially (Nader *et al*, 2000; Debiec and Ledoux, 2004; Ben Mamou *et al*, 2006; Jin *et al*, 2007). Indeed, it is accepted in the literature that the lack of spontaneous recovery, the selectivity to reactivated memories, and the presence of intact short-term memory are criteria that define the reconsolidation process. Taken together, our results suggest that the effect of clonidine on memory is mediated by reconsolidation blockade.

Clinical Relevance

Currently, there are no specific pharmacological approaches to treat PTSD symptoms. Therefore, there is a need for preclinical research to identify new candidate drugs and to develop novel therapeutic interventions. The present study has implications for the potential clinical use of reconsolidation blockade by clonidine. First, we determined that the dose of 100 μg/kg optimally disrupts fear memory retention in both male and female rats. Conversion from the animal dose to a human equivalent dose in mg/kg may be obtained by applying a formula that takes the body surface area into account. With this calculation, our animal dosage of 100 μg/kg translates into a dose of 1.135 mg for a 70-kg person (Reagan-Shaw *et al*, 2008). Such a dose is well within the safe range for daily human use that has a maximum of 2.4 mg (Physician's Desk Reference; http://www.pdr.net). Nevertheless, as clonidine is known to induce hypotension, patients being treated with clonidine should be medically monitored. We also found that clonidine-induced memory impairments are selective to the reactivated memory. Thus, we can hypothesize that using clonidine in combination with traumatic memory reactivation will decrease the intensity of that memory without disrupting other unrelated memories. Additionally, we observed that postreactivation clonidine does not affect learning of new fear memories, implying that patients would be able to experience and remember new events normally. These are all valuable aspects for clinical use, as optimal treatments should be specific and not interfere with other processes (Steckler and Risbrough, 2011).

Clonidine has been found to improve symptoms such as hyperarousal (Harmon and Riggs, 1996; Donnelly, 2003), impulsivity (Donnelly, 2003) (Viola *et al*, 1997), and nightmares (Kinzie and Leung, 1989; Kinzie *et al*, 1994) when administered chronically to patients. However, some experienced a return of symptoms upon termination of treatment (Porter and Bell, 1999), and the possibility that the beneficial effects would decrease over time remains. A significant advantage of reconsolidation blockade by clonidine in treating PTSD symptoms would be that it does not require chronic administration of the drug, as based upon our animal findings the maximal effect would probably be obtained within a few sessions. Consequently, this would make lasting side effects unlikely. Furthermore, we showed that memory disruption following postretrieval clonidine is long lasting; thus, it is reasonable to hope that combining memory reactivation with clonidine administration could permanently weaken PTSD symptoms such as intrusive memories without the possibility of relapse.

Although fear conditioning models the enhanced fear response upon recollection of the traumatic event, this is only one of the many pathophysiological and behavioral characteristics of PTSD. Nightmares, avoidance, and hyperarousal are common, and alterations of several neurotransmitter systems have also been observed. Further investigations will be necessary to verify whether clonidine can improve other aspects of this complex pathology in an animal model.

In conclusion, results of this study demonstrate that systemic administration of clonidine after retrieval persistently weakens fear memories through reconsolidation blockade. We show that this effect is maximal after two treatments, is present in both male and female rats, is selective to the reconsolidation time window and to reactivated memories, and does not affect further fear learning. These preclinical findings indicate potential to further develop clinical approaches using clonidine as a reconsolidation blocker in the treatment of PTSD symptoms.

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DISCLOSURE

The authors declare no conflict of interest.

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Learning and reconsolidation implicate different synaptic mechanisms

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Synaptic mechanisms underlying memory reconsolidation after retrieval are largely unknown. Here we report that synapses in projections to the lateral nucleus of the amygdala implicated in auditory fear conditioning, which are potentiated by learning, enter a labile state after memory reactivation, and must be restabilized through a postsynaptic mechanism implicating the mammalian target of rapamycin kinase-dependent signaling. Fear-conditioning–induced synaptic enhancements were primarily presynaptic in origin. Reconsolidation blockade with rapamycin, inhibiting mammalian target of rapamycin kinase activity, suppressed synaptic potentiation in slices from fear-conditioned rats. Surprisingly, this reduction of synaptic efficacy was mediated by post- but not presynaptic mechanisms. These findings suggest that different plasticity rules may apply to the processes underlying the acquisition of original fear memory and postreactivational stabilization of fear-conditioning–induced synaptic enhancements mediating fear memory reconsolidation.

Newly formed memories are stabilized over several hours after their acquisition for long-term storage. This protein synthesis-dependent process, termed cellular consolidation (1), critically depends on the permanence of acquisition-induced synaptic modifications (2). Once retrieved, consolidated memory returns to an unstable state and must be restabilized/reconsolidated to persist (3–8). Reconsolidation, which is also a protein synthesis-dependent process, has been observed across many behavioral paradigms, and reported for a range of species (9–12), including humans (13). Mechanistically, reconsolidation blockade differs from extinction of conditioned fear memory, also resulting in diminished fear responses, as these behavioral processes are mediated by distinct neurochemical mechanisms (14).

To date, studies of consolidation have typically reported that the molecular and cellular changes induced by learning are prevented when this memory process is inhibited (2, 15). Thus, synaptic growth was enhanced by long-term sensitization in *Aplysia californica* (16), whereas blockade of consolidation of this trace with either RNA or protein synthesis inhibitors prevented the stabilization of the morphological correlates of memory changes (17). Similarly, blockade of reconsolidation has also been shown to reverse the molecular (18) and cellular (6) modifications induced by memory reactivation. Although both the memory acquisition and consolidation processes were studied previously at the level of synaptic functions (2), synaptic mechanisms of reconsolidation are largely unknown. Thus, we asked whether reconsolidation blockade reverses learning-induced synaptic plasticity, and, if so, how such modifications of synaptic mechanisms in the circuits for a learned behavior might be mediated.

In this study, we tested the hypothesis that synaptic enhancements induced by fear learning are reversed by reconsolidation blockade, using systemic injections of rapamycin that inhibits mammalian target of rapamycin (mTOR) kinase activity. mTOR kinase regulates protein synthesis at the translational level and is critical for fear memory reconsolidation (19–22). We found that fear learning-induced enhancements of synaptic efficacy were predominantly presynaptic in origin. However, although the impairment in reconsolidation reversed learning-induced synaptic enhancements, this was accomplished by changes in postsynaptic functions. These findings indicate that stabilization of fear-conditioning–associated synaptic enhancements after retrieval recruits a form of synaptic plasticity that is different from synaptic modifications induced during the acquisition of original memory, thereby revealing a distinct mechanism mediating memory reconsolidation.

Results

Fear Conditioning Is Associated with Potentiation of Synaptic Transmission in Cortical and Thalamic Inputs to the Lateral Amygdala. To explore synaptic mechanisms of memory reconsolidation, we trained male Sprague-Dawley rats in a classical single-trial auditory fear conditioning paradigm by pairing a tone [conditioned stimulus (CS)] with a footshock [unconditioned stimulus (US)] (23, 24). Rats in the paired (CS–US) group demonstrated more freezing than control rats (CS-only or US-only groups) in response to the CS during a long-term memory test [postreactivation long-term memory (PR-LTM)] (Fig. 1 *A* and *B*; two-way ANOVA, $P < 0.001$; post hoc Bonferroni's simultaneous multiple comparisons revealed significant differences between paired and CS-only groups, $P < 0.001$, and paired and US-only groups, $P < 0.001$, but no differences between CS-only and US-only groups, $P = 1.0$). We found also that single CS presentations during memory reactivation did not produce fear extinction under our experimental conditions, as the amount of freezing in fear-conditioned rats at PR-LTM1 was not different from that at PR-LTM2 measured 24 h later (Fig. 1*C*; *t* test, $P = 0.75$ for PR-LTM1 versus PR-LTM2).

We examined the effects of fear learning on synaptic transmission in the CS pathways, performing whole-cell patch-clamp recordings from visualized neurons in slices of the amygdala obtained from paired, CS-only, US-only and behaviorally naive (naïve) rats. At 48 h postconditioning, we recorded glutamatergic excitatory postsynaptic currents (EPSCs) evoked in lateral amygdala (LA) neurons under voltage-clamp conditions with stimulating electrodes placed to activate either thalamic input (internal capsule) or cortical input (external capsule) to the LA (25). These two projections deliver the auditory CS information to the LA during fear conditioning (23). Consistent with the role of synaptic enhancements in the CS pathways in retention of fear memory (26–31), we found that synaptic strength, as reflected in input–output curves, was significantly increased in both thalamic and cortical inputs to the LA in slices from paired, compared with the CS-only, US-only, and naïve control groups (Fig. 1 *D* and *E*). There were no differences in synaptic input–output curves in thalamo-LA or cortico-LA projections among the control groups, indicating that

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The authors declare no conflict of interest.

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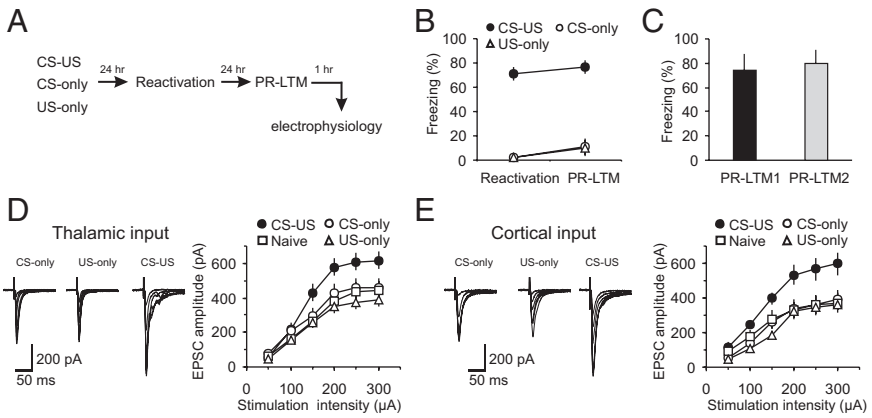

Fig. 1. Fear conditioning leads to synaptic enhancements in cortical and thalamic inputs to the LA. (*A*) A schematic representation of the experimental design. Rats were trained in a single-trial fear conditioning paradigm and tested at 24 h (PR-LTM) after reactivation trials. (*B*) Percent freezing observed in fear-conditioned rats (CS–US, paired) and in rats that received CS or US only (CS–US, $n = 22$ rats; CS-only, $n = 20$ rats; US-only, $n = 6$ rats). There were no differences between freezing responses at reactivation and PR-LTM in the CS–US ($P = 0.47$), CS-only ($P = 0.15$), or US-only ($P = 0.35$) groups. (*C*) Percent freezing observed in CS–US rats at PR-LTM1 (a first reactivation trial) and PR-LTM2 (a second memory test performed 24 h after PR-LTM1) ($n = 5$ rats; paired t test, $P = 0.51$ for PR-LTM1 versus PR-LTM2). (*D*, *Left*) Averaged EPSCs evoked in thalamic input to the LA by presynaptic stimuli of increasing intensity in slices from naïve (10 rats), CS-only, US-only, and paired groups of rats. Traces are averages of 10 EPSCs. (*D*, *Right*) Synaptic input–output curves obtained in thalamic input to the LA (naïve, $n = 26$ neurons; CS-only, $n = 16$ neurons; US-only = 12 neurons; paired, $n = 14$ neurons). Peak amplitudes of the EPSCs were significantly different between naïve, CS-only, US-only, and paired groups (two-way ANOVA, $P < 0.001$). Post hoc Bonferroni's simultaneous multiple comparisons revealed significant differences in the EPSC amplitudes between naïve and paired groups ($P < 0.001$), between CS-only and paired groups ($P < 0.01$), and between US-only and paired groups ($P < 0.001$). Thus, synaptic strength in thalamic input was enhanced in fear conditioned rats (paired group). (*E*) In cortical input, peak amplitudes of the EPSCs also differed significantly between naïve ($n = 16$), CS-only ($n = 8$), US-only ($n = 12$), and paired ($n = 12$) groups (two-way ANOVA, $P < 0.001$). EPSC amplitudes were larger in the paired group compared with either naïve ($P < 0.001$), CS-only ($P < 0.001$), or US-only group ($P < 0.001$; Bonferroni's simultaneous multiple comparisons). Results are shown as means ± SEM.

neither the CS or US alone nor exposure of rats to the training context produced detectable synaptic modifications.

Fear-Conditioning–Induced Synaptic Potentiation Is Suppressed Following Reconsolidation Blockade. We asked whether reconsolidation blockade with rapamycin, an efficient blocker of mTOR kinase activity (22, 32), would reverse learning-induced enhancements in synaptic efficacy in thalamo-LA and cortico-LA projections. Memory reactivation entailed presentation of a single CS (24 h postconditioning), after which rats received an injection of either rapamycin (20 mg/kg, i.p.; RAP) or vehicle (VEH). It has been demonstrated previously that systemic administration of rapamycin, in doses that impair memory reconsolidation and are comparable to those used in our experiments, did not result in any unspecific alterations in behavior, including anxiety levels, foot shock sensitivity, flinch and vocalization thresholds (20). Whereas both groups showed comparable levels of conditioned freezing during reactivation, rapamycin-treated rats showed lesser freezing 24 h later (indicative of impaired PR-LTM) compared with both the vehicle group (t test, $P = 0.023$) and with the original fear response in same rats during the reactivation session (paired t test, $P = 0.038$; Fig. 2 *A* and *B*). The inhibitory action of rapamycin on

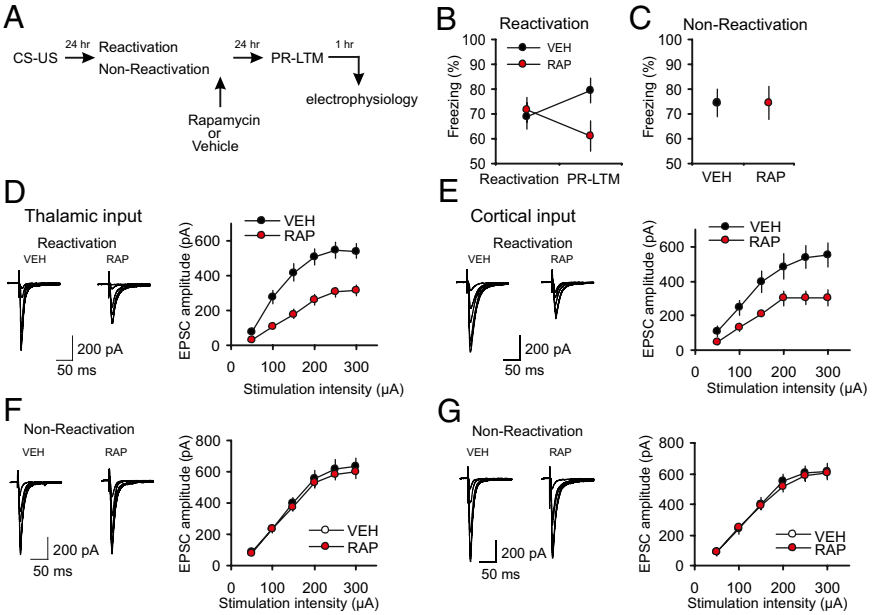

Fig. 2. Postretrieval rapamycin impairs reconsolidation of fear memory and suppresses conditioning-induced synaptic enhancements. (*A*) A schematic representation of the experiments where fear-conditioned rats received a postretrieval injection of rapamycin (RAP; 20 mg/kg, i.p.) or vehicle (VEH). (*B*) There was no significant difference in percent freezing between VEH-treated ($n = 29$) and RAP-treated ($n = 29$) rats during memory reactivation (t test, $P = 0.74$). The difference in freezing between reactivation and PR-LTM tests in the VEH group did not reach the level of statistical significance ($P = 0.06$). A significant impairment was observed in RAP rats during the PR-LTM test (see text for details). (*C*) Rapamycin had no effect on conditioned freezing in "nonreactivated" control rats. Rats in nonreactivation group received rapamycin or vehicle injections at 24 h postconditioning without memory reactivation and PR-LTM was tested 24 h after the injections (RAP, $n = 16$ rats; VEH, $n = 8$ rats; t test, $P = 0.9$ for VEH group vs. RAP group). (*D*, *Left*) Averaged EPSCs evoked in thalamic input to the LA by stimuli of increasing intensity in slices from fear-conditioned rats which received postreactivation injections of VEH or RAP. (*D, Right*) Synaptic input–output curves obtained in thalamic input in slices from both groups of rats (VEH, $n = 12$ neurons; RAP, $n = 13$ neurons (two-way ANOVA, $P < 0.001$ for VEH group versus RAP group of conditioned rats). (*E*) Experiments were analogous to *D*, but the EPSCs were recorded in cortical input to the LA (VEH, $n = 12$ neurons; RAP, $n = 8$ neurons; two-way ANOVA, $P < 0.001$). (*F*) Rapamycin or vehicle were injected at 24 h postconditioning without memory reactivation and synaptic input–output curves were obtained in thalamic input 24 h after the injections (VEH, $n = 14$ neurons; RAP, $n = 23$ neurons; two-way ANOVA, $P = 0.275$). (*G*) Experiments were analogous to *F* but the EPSCs were recorded in cortical input (VEH, $n = 9$ neurons; RAP, $n = 19$ neurons; two-way ANOVA, $P = 0.515$). Results are shown as means ± SEM.

conditioned freezing was not observed when reactivation session was omitted (nonreactivation control groups: rats that received rapamycin or vehicle injections without a prior memory reactivation; Fig. 2*C*, *t* test, $P = 0.9$ for VEH group versus RAP group). The latter finding indicates that the effect of rapamycin might be specific to its ability to suppress fear memory reconsolidation and was not due to the unspecific lasting effects on fear memory retrieval. Consistent with this notion, retrieval of conditioned fear memory was shown to be unaffected by rapamycin injected 30 min before memory reactivation (21).

The observed decreases in conditioned freezing in rats which received rapamycin injections were associated with a rightward shift in the input–output curves in both thalamic and cortical inputs to the LA, compared with vehicle-injected rats, indicating a decrease in synaptic strength that had been previously increased by fear conditioning (Fig. 2 *D* and *E*). In contrast, synaptic strength remained enhanced in both auditory inputs to the LA in rapamycin-injected but nonreactivated rats (Fig. 2 *F* and *G*). Overall, these findings demonstrate the requirement for mTOR activity in maintaining the postreactivation stability of synaptic potentiation in the conditioned stimulus pathways.

Synaptic Mechanisms of Fear Learning and Reconsolidation. What are the loci (pre- versus postsynaptic) of synaptic enhancements after learning, compared with those involved in synaptic modifications after reconsolidation blockade? Efficacy of synaptic transmission is determined by the probability of neurotransmitter (glutamate) release (P_r) and/or postsynaptic responsiveness to glutamate contained in single synaptic vesicles (quantal amplitude), as well as by the number of effective synapses (33). We therefore estimated P_r and quantal amplitude in both thalamic and cortical inputs to the LA following fear conditioning and postreactivation rapamycin treatment. In agreement with previous findings (26, 28), we found that the increase in synaptic strength in fear-conditioned rats (as shown in Fig. 1 *D* and *E*) was accompanied by a decrease in the magnitude of paired-pulse ratio (PPR) recorded at a 50-ms interstimulus interval in both studied pathways, compared with control rats (Fig. 3 *A*–*C*). Because the magnitude of PPR varies inversely with the basal P_r (ref. 34; but see ref. 35), the observed increases in synaptic efficacy in the CS pathways of conditioned rats appear at least in part be due to the higher P_r. To estimate postsynaptic responsiveness, we recorded asynchronous single-quanta synaptic events evoked by stimulation of either thalamic or cortical inputs in the external medium where strontium (Sr^{2+}) was substituted for Ca^{2+} (25). Asynchronous EPSCs may be observed for hundreds of milliseconds following the presynaptic stimulation pulse, thus permitting analysis of quantal responses in specific projections to the target area (36). Surprisingly, the acquisition of conditioned fear memory did not lead to detectable changes in the amplitude of single-quantum EPSCs in either thalamic (Fig. 3 *D* and *E*) or cortical inputs compared with the CS-only group (Fig. 3 *F* and *G*), which suggests a lack of postsynaptic modifications under present conditions (37).

To explore further the possibility of postsynaptic modifications in the CS pathways during the single-trial fear-conditioning, we recorded AMPA receptor (AMPAR) EPSCs in both cortical and thalamic inputs to the LA in slices from the CS–US and CS-only groups at holding potentials of −70 mV or +40 mV. In these experiments, the intrapipette recording solution contained spermine (200 μM), a naturally occurring polyamine. We then calculated the rectification index for AMPAR EPSCs at cortico-LA and thalamo-LA synapses in slices from both behavioral groups, dividing the amplitude of AMPAR EPSC at −70 mV by the EPSC amplitude at +40 mV (as in ref. 30). Modifications in this index are indicative of changes in the AMPA receptor subunit composition. Specifically, the GluR1 subunit trafficking to synapses is normally

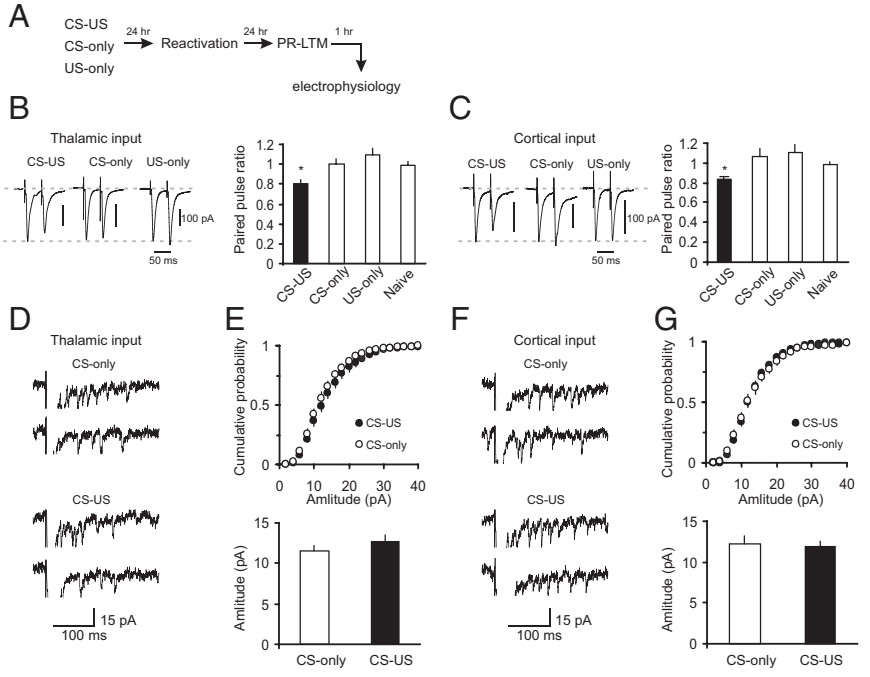

Fig. 3. Fear-conditioning–induced synaptic strengthening in inputs to the LA is primarily presynaptically mediated. (*A*) A schematic representation of the experimental design. Rats were trained in a single-trial fear conditioning paradigm and tested at 24 h (PR-LTM) after reactivation trials. (*B*, *Left*) Examples of EPSCs evoked in thalamic input to the LA with paired presynaptic stimuli in slices from CS-only, US-only, and fear-conditioned (CS–US) rats. The interstimulus interval was 50 ms. Traces are averages of 10 paired EPSCs. (*B*, *Right*) Summary plot of the paired-pulse stimulation experiments. Paired pulse ratio (PPR) was calculated as the ratio of the second EPSC amplitude to the first EPSC amplitude. CS-only group of rats, $n = 10$ neurons; US-only group, $n = 12$ neurons; naïve group, $n = 17$ neurons; CS–US group, $n = 9$ neurons. The magnitude of PPR in the paired group of rats (CS–US) was significantly decreased compared with naïve, CS-only, or US-only rats (one-way ANOVA, $F_{3,44} = 4.02$, $P = 0.013$. There was no difference in PPR values between naïve and CS-only ($P = 0.45$) or US-only groups ($P = 0.203$). All electrophysiological recordings for Fig. 3 were performed at 48 h post-CS–US pairing or single CS or US presentations (24 h postreactivation). (*C*) Experiments were analogous to *B*, but the EPSCs were recorded in cortical input to the LA. CS-only group, $n = 8$ neurons; US-only group, $n = 9$ neurons; naïve group, $n = 18$ neurons; paired group, $n = 7$ neurons. The magnitude of PPR in the paired group was significantly decreased compared with naïve, CS-only, or US-only rats (one-way ANOVA, $F_{3,38} = 3.37$, $P = 0.028$). There was no difference between naïve and CS-only rats ($P = 0.1$) or US-only rats ($P = 0.1$). (*D*) Traces of the asynchronous quantal EPSCs evoked by stimulation of thalamic input (V_H= −70 mV) in slices from the CS-only and paired rats. In these experiments, Sr^{2+} was substituted for extracellular Ca^{2+}. (*E*, *Upper*) Cumulative amplitude histograms of asynchronous quantal events recorded in thalamic input to the LA in slices from the CS-only and paired groups. (*E*, *Lower*) Summary plot of asynchronous EPSCs data (mean amplitude; CS-only, $n = 9$ neurons; paired, $n = 10$ neurons; *t* test, $P = 0.34$). (*F* and *G*) Experiments were analogous to *D* and *E*, but the asynchronous EPSCs were recorded in cortical input to the LA (CS-only, $n = 5$ neurons; paired, $n = 7$ neurons; *t* test, $P = 0.73$). Error bars indicate SEM.

expected to increase the rectification index (30). In our experiments, the values of rectification index, calculated at PR-LTM test, were not different between the CS-only and CS–US groups (Fig. 4 *A–D*). The observed lack of changes in rectification index, at times when consolidated fear memory was assayed, indicates that fear memory consolidation under conditions of the single-trial fear conditioning did not implicate increased GluR1 trafficking at activated synapses.

In contrast, we did not observe changes in the PPR magnitude in rats that received postretrieval injections of rapamycin, compared with the vehicle group (Fig. 5 *A–D*). Moreover, the magnitude of postretrieval PPR in rapamycin-treated rats did not differ from that in the paired group that did not receive the rapamycin treatment (as shown in Fig. 3 *B* and *C*; *t* test, $P = 0.39$ and $P = 0.37$ between groups in thalamic and cortical inputs, respectively), suggesting that presynaptic enhancements associated with fear conditioning were retained following reconsolidation blockade. Confirming that rapamycin had no direct effects on synaptic plasticity associated with the acquisition of conditioned fear memory, the magnitude of PPR in nonreactivated rats was also unaffected by rapamycin (Fig. 5 *A–D*). However, the amplitude of single-quantum thalamo-LA or cortico-LA EPSCs was significantly decreased in slices from rats with an impairment in reconsolidation, compared with the vehicle-injected rats (Fig. 5 *E–H*). Notably, postretrieval reconsolidation itself had no effect on the quantal amplitude. Thus, we compared the quantal amplitude values in thalamic and cortical inputs in the VEH group in Fig. 5 *F* and *H*, where fear memory was reactivated, with the quantal amplitude in the CS-only group in Fig. 3 *E* and *G*, respectively, where no reconsolidation was present as fear memory was not formed. The amplitude of unitary EPSCs did not differ between the groups (thalamic input: CS-only group in Fig. 3*E* versus VEH group in Fig. 5*F*, *t* test, $P = 0.98$; cortical input: CS-only group in Fig. 3*G* versus VEH group in Fig. 5*H*, *t* test, $P = 0.36$). Taken together, our results suggest that mTOR-dependent reconsolidation of fear memory and stabilization of conditioning-produced synaptic enhancements in CS pathways may implicate the mechanisms of postsynaptic plasticity, preventing decreases in the postsynaptic responsiveness to glutamate.

Discussion

Our findings demonstrate that retrieval of fear memory converts learning-induced synaptic modifications to a labile state. Although retrieval, presumably, triggers the mechanisms of extinction learning in addition to reconsolidation of the original fear memory, augmentation of extinction following rapamycin treatment is an unlikely explanation for our results because extinction is blocked by inhibition of protein synthesis, not promoted by it (38). The cellular processes that maintain increased synaptic strength in the CS pathways after a memory recall require mTOR kinase activity. If mTOR signaling-dependent reconsolidation is blocked, synaptic strength returns to the default (preconditioning) level. Reconsolidation likely resulted from a form of synaptic plasticity that is mechanistically distinct from that involved in the acquisition of conditioned fear memory. Specifically, the decreases in synaptic strength, which we observed following the disruption of reconsolidation by rapamycin, appear due to modifications in postsynaptic processes, rather than reversal of presynaptic enhancements produced by initial fear learning. In our experiments, a single CS–US pairing was associated with increased P_r in auditory inputs to the LA. It is possible that multiple CS–US pairings would recruit postsynaptic mechanisms during the memory acquisition (as in ref. 30). The finding that the fear learning-induced enhancements in presynaptic function were retained following reconsolidation blockade, whereas postsynaptic restabilization of synaptic transmission was required to sustain its potentiation, indicates the potential role for both pre- and postsynaptic plasticity in maintaining conditioned fear memory after retrieval. The observed dissociation of the mechanisms used to enhance synaptic efficacy during learning and those affected by reconsolidation implies that reconsolidation might be not a unitary process from the cellular and molecular prospective.

These results, however, do not exclude a possibility that there might be different rules determining whether pre- and postsynaptic mechanisms are recruited during reconsolidation. One scenario is that the presynaptic mechanisms do not undergo reconsolidation and retained as a molecular and cellular legacy of prior learning. Alternatively, the presynaptic mechanisms may undergo reconsolidation, but the molecular pathways mediating presynaptic reconsolidation do not require mTOR activity. Another possibility might be that the postretrieval rapamycin administration might uncover or trigger a certain postsynaptic

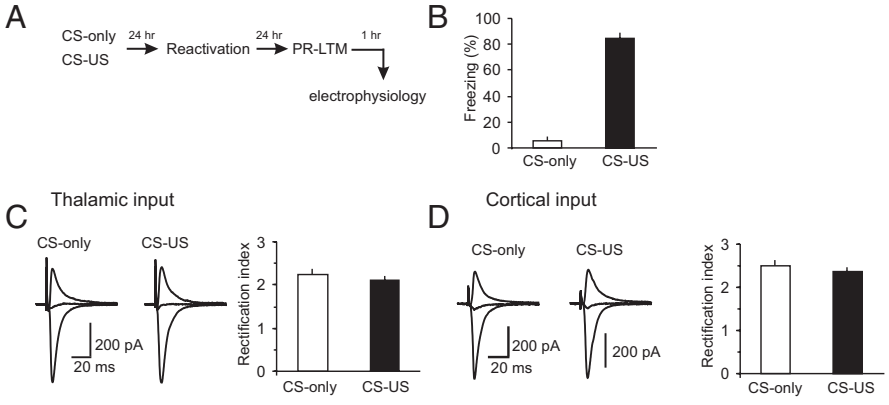

Fig. 4. Rectification index for AMPAR EPSCs in inputs to the LA is not affected by single-trial fear conditioning. (*A*) A schematic representation of the experimental design. (*B*) Percent freezing observed in fear-conditioned rats (CS–US group) and CS-only rats at PR-LTM test (CS–US, $n = 5$ rats; CS-only, $n = 6$ rats; $P < 0.001$ between the groups). (*C*, *Left*) Averaged AMPAR EPSCs (15 traces) recorded in thalamic input to the LA at holding potentials of −70 mV, 0 mV, and +40 mV in slices from CS–US or CS-only rats. The AMPAR EPSCs were recorded in the presence of the NMDAR antagonist D-AP5 (50 μM). Intrapipette recording solution contained spermine (200 μM). The intensity of presynaptic stimulation was adjusted to produce the EPSCs of approximately same amplitude in both behavioral groups at a holding potential of −70 mV. (*C*, *Right*) the rectification index values at the thalamo-LA synapses in slices from CS–US and CS-only groups (CS–US group, $n = 19$ neurons from five rats; CS-only group, $n = 23$ neurons from six rats; $P = 0.44$ between two groups). (*D*) Experiments were analogous to *C* but the EPSCs were recorded in cortical input to the LA (CS–US group, $n = 16$ neurons from five rats; CS-only group, $n = 22$ neurons from six rats; $P = 0.4$ between two groups). Error bars indicate SEM.

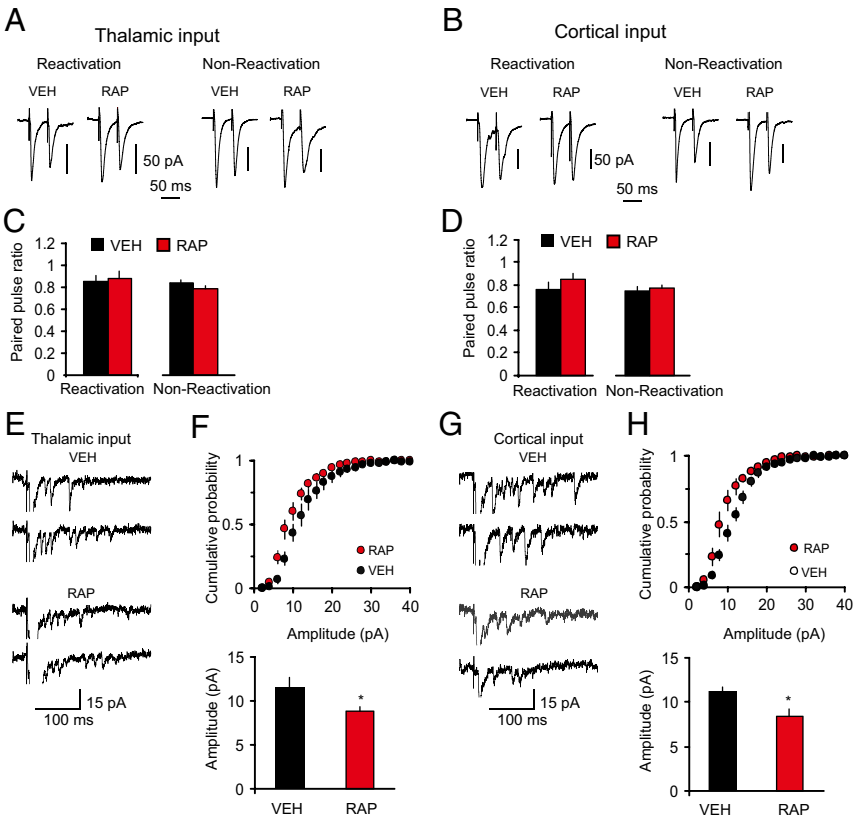

Fig. 5. Postretrieval stabilization of conditioning-induced potentiation in inputs to the LA implicates postsynaptic mechanisms. (*A*, *Left*) Reactivation, examples of EPSCs evoked in thalamic input to the LA with paired stimuli in slices from fear-conditioned rats that received one injection of either rapamycin (RAP; 20 mg/kg, i.p.) or vehicle (VEH) immediately after the fear memory reactivation (memory was retrieved at 24 h postconditioning). Recordings were performed 24 h after the memory reactivation. (*A*, *Right*) Nonreactivation, examples of EPSCs recorded in slices from rats that received rapamycin or vehicle injections at 24 h postconditioning without memory reactivation. Recordings were performed 24 h after the injections. (*B*) Analogous to *A*, but the EPSCs were recorded in cortical input. (*C*) Summary plot of PPR data in thalamic input (reactivation: VEH, $n = 19$ neurons; RAP, $n = 21$ neurons; t test, $P = 0.79$; nonreactivation: VEH, $n = 17$ neurons; RAP, $n = 24$ neurons; t test, $P = 0.19$). (*D*) Summary plot of PPR data in cortical input (reactivation: VEH, $n = 11$ neurons; RAP, $n = 13$ neurons; t test, $P = 0.31$; nonreactivation: VEH, $n = 10$ neurons; RAP, $n = 19$ neurons; t test, $P = 0.63$). (*E*) Traces of the asynchronous quantal EPSCs evoked by stimulation of thalamic input in slices from VEH or RAP groups. (*F*, *Upper*) Cumulative amplitude histograms of asynchronous quantal events recorded in thalamic input to the LA in slices from VEH or RAP rats. (*F*, *Lower*) Summary plot of asynchronous EPSCs data (mean amplitude; VEH, $n = 5$ neurons; RAP, $n = 7$ neurons; t test, $^*P = 0.048$). (*G* and *H*) The experiments were analogous to *E* and *F*, but the asynchronous EPSCs were recorded in cortical input to the LA (VEH, $n = 5$ neurons; RAP, $n = 6$ neurons; t test, $^*P = 0.026$). Error bars indicate SEM.

process reducing unitary events amplitude through the mechanisms not related to memory reconsolidation. The latter possibility is, however, unlikely as the effects of rapamycin were specifically linked to reactivation of consolidated fear memory. It would be interesting to examine in future studies whether postreactivation infusions of compounds (when they become available) that block specifically presynaptic mechanisms of memory consolidation could also suppress reconsolidation. Moreover, certain experimental characteristics, including the intensity of training procedures or memory age, could also determine whether and how consolidation and reconsolidation occur (7). Thus, although presynaptic mechanisms did not undergo reconsolidation under present experimental conditions, it might be possible that, under other conditions (e.g., with a stronger training protocol), the presynaptic mechanisms could become susceptible to reconsolidation.

Although postretrieval rapamycin virtually completely reversed the postconditioning enhancement in thalamo-LA and cortico-LA EPSCs produced by fear conditioning, it produced only a partial reduction in learned freezing. This divergence between electrophysiological and behavioral results suggests first, that there might be other mechanisms besides synaptic enhancement in CS pathways to the LA that underlie fear learning, and second that these other mechanisms do not require mTOR activity for maintaining their stability, thus warranting future investigation.

Further experiments will be required to identify other molecular components, both upstream and downstream, implicated in the mTOR-dependent control of fear memory reconsolidation at synaptic level and differentiate between the above-described hypotheses. Regardless, our findings suggest that targeting the mechanisms underlying postretrieval stabilization of synaptic plasticity could potentially be used to alleviate symptoms of anxiety disorders in which conditioned fear plays a role, such as posttraumatic stress disorder (PTSD) (39).

Experimental Procedures

Behavior: Single-Trial Fear Conditioning. All animal procedures were approved by McLean Hospital's Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (350–375 g) were housed for at least one week before the experiment. Before behavioral training, rats were assigned randomly to one of four groups: paired (CS–US), CS-only, US-only, and naïve. On the training day, rats from the paired group were placed into a conditioning chamber, housed within a sound-attenuating cabinet (Med Associates), for 2 min before the onset of the CS. The CS was a tone (5 kHz, 75 dB) that lasted for 30 s. The last 2 s of the CS were paired with a continuous foot shock (0.6 mA, the US). After additional 30 s in the chamber, the rat was returned to its home cage. Memory was reactivated 24 h after training. Rats were then

tested 24 h later for PR-LTM. For all tests, rats were placed into a different context and, 2 min later, exposed to the tone CS (5 kHz, 75 dB) for 60 s. Thirty sec after the termination of the tone, they were removed from the chamber and returned to their home cage. Freezing scores were calculated as the percentage of the total CS duration that the rat remained immobile (frozen) other than breathing. The identical training protocol has been used previously to demonstrate that auditory fear conditioning can undergo reconsolidation after retrieval that was blocked by BLA infusions of anisomycin (3). Rats in the CS-only group were trained and tested similarly to those of the paired CS–US group except that the foot shock US was omitted during training. Rats in the behaviorally naïve group were handled but not exposed to either training or testing chambers. Rats in the US-only group were placed into the chamber where they received a continuous foot shock (0.6 mA, 2 s) without any delay and then immediately removed from the chamber. Under these training conditions, the contribution of contextual fear learning was minimized. Responses of US-only rats to the tone CS (5 kHz, 75 dB) for 60 s were tested 24 h later in a different context and retested the next day (24 h later). Immediately after the conclusion of the PR-LTM session, rats were killed and brain slices containing the amygdala were prepared for electrophysiological recordings. In the experiments testing the effects of mTOR blockade on fear memory reconsolidation, rapamycin (20 mg/kg; LC Laboratories) or vehicle [70% DMSO (700 mg/mL)] was injected i.p. immediately after the fear memory reactivation. Freezing responses were evaluated 24 h later, and rats were used for electrophysiological recordings immediately after that. For statistical analysis, we used either a Student *t* test or two-way ANOVA with post hoc Bonferroni's simultaneous multiple comparisons or one-way ANOVA ($P < 0.05$ was considered significant). The comparisons between slices from different experimental groups of rats were performed blind.

Electrophysiological Recordings. Slices of the amygdale (both left and right, 250–300 μm) were prepared from behaviorally trained and naïve rats (as described above) with a vibratome. Slices were continuously superfused in solution containing 119 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 26.0 mM $NaHCO_3$, 10 mM glucose, and 0.05 mM picrotoxin and equilibrated with 95% O_2 and 5% CO_2 (pH 7.3–7.4) at room temperature (22–24 °C). Whole-cell recordings of compound EPSCs were obtained from pyramidal neurons in the lateral nucleus of the amygdala (LA) under visual guidance (DIC/infrared optics) with an EPC-10 amplifier and Pulse v8.67 software (HEKA Elektronik). Evoked synaptic responses were triggered by field stimulation of the internal capsule (thalamic input) or the external capsule (cortical input) at 0.05 Hz with a fine-tipped (~2 mm) glass stimulation pipette. The recording patch electrodes (3–6 MΩ resistance) contained 120 mM Cs-methane-sulfonate, 5 mM NaCl, 1 mM $MgCl_2$, 10 mM BAPTA, 10 mM Hepes, 2 mM MgATP, and 0.1 mM NaGTP (adjusted to pH 7.2 with CsOH). A high concentration of the Ca^{2+} chelator BAPTA was included in the pipette solution to prevent the induction of synaptic plasticity in the studied pathways in slices which is not related to the modifications induced by behavioral training. Currents were filtered at 1 kHz and digitized at 5 kHz. The AMPAR EPSC amplitude was measured at a holding potential of −70 mV as the difference between the mean current during a prestimulus baseline and the mean current over a 1- to 2-ms window at the response peak. The evoked asynchronous EPSCs were recorded in both thalamic and cortical inputs to the LA in the Sr^{2+}-containing external solution and analyzed using Mini Analysis Program (Synaptosoft).

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Systemic Mifepristone Blocks Reconsolidation of Cue-Conditioned Fear; Propranolol Prevents This Effect

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Reducing reconsolidation of reactivated traumatic memories may offer a novel pharmacological treatment for posttraumatic stress disorder (PTSD). Preclinical research is needed to identify candidate drugs. We evaluated the ability of postreactivation mifepristone (RU38486, a glucocorticoid antagonist), alone and in combination with propranolol (a beta-adrenergic blocker), both given systemically, to reduce cue-conditioned fear in rats. On Day 1, a 30-s tone conditioned stimulus (CS) was paired with an electric shock unconditioned stimulus (US). On Day 2, the CS was presented without the US (reactivation), and the freezing conditioned response (CR) was measured. This was immediately followed by subcutaneous injection of vehicle, mifepristone 30 mg/kg, propranolol 10 mg/kg, or both. On Day 3, the CR was again measured as a test of postreactivation long-term memory (PR-LTM). On Day 10, the CR was again measured to evaluate spontaneous recovery. On Day 11, the US was presented alone (reinstatement). On Day 12, the CR was again measured. A fifth group received mifepristone without the CS presentation (nonreactivation) on Day 2. A sixth group was tested four hours after the Day 2 mifepristone injection to measure postreactivation short-term memory. Postreactivation, but not nonreactivation, mifepristone produced a decrement in the CR that did not undergo spontaneous recovery and underwent only modest reinstatement. Mifepristone did not exert its effect when administered concurrently with propranolol. Postreactivation mifepristone did not impair short-term memory. Systemic mifepristone blocks the reconsolidation of cue-conditioned fear in rats. Concurrent administration of propranolol prevents this effect. Postreactivation mifepristone may be a promising treatment for PTSD, but not necessarily in combination with propranolol.

Keywords: memory, conditioning, classical, fear, mifepristone, propranolol (all MeSH terms)

Reconsolidation is a memory process that has been studied largely during the last decade. It has long been recognized that when something is first learned, for example a conditioned fear response, its trace exists in an unstable state in the brain. In order for its memory to be retained, it must be converted to a stable state through a process known as consolidation. Reconsolidation theory holds that when the stabilized memory is reactivated (retrieved) under certain circumstances, it returns to an unstable state, from which it must be reconsolidated if it is to endure (Nader & Hardt, 2009). The reconsolidation process has mainly been revealed through its blockade. When certain drugs are administered shortly after reactivation, subsequent testing finds the memory to be diminished (Abrari, Rashidy-Pour, Semnanian, & Fathollahi, 2008; Debiec & Ledoux, 2004; Jin, Lu, Yang, Ma, & Li, 2007; Muravieva & Alberini, 2010; Nader, Schafe, & Le Doux, 2000; Przybyslawski, Roullet, & Sara, 1999; Taubenfeld, Riceberg, New, & Alberini, 2009).

In contrast to reconsolidation, extinction is a process whereby new learning inhibits the expression of old learning, for example, learning to no longer fear a previously feared object or situation (Milad, Rauch, Pitman, & Quirk, 2006; Quirk & Mueller, 2008). Although the original learning is no longer behaviorally evident, its continuing presence is revealed under certain circumstances. An

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extinguished behavior may return with the passage of time (spontaneous recovery; Quirk, 2002). It may also become evident when testing takes place in a context other than that in which it was extinguished (renewal), and it may be made to return by readministration of the unconditioned stimulus (US) alone (reinstatement; Bouton, 2002). Because memories that have undergone reconsolidation blockade putatively do not undergo spontaneous recovery (Bustos, Maldonado, & Molina, 2006; Duvarci & Nader, 2004; Lin, Mao, & Gean, 2006; Jin et al., 2007), renewal (Duvarci & Nader, 2004), or reinstatement (Bustos et al., 2006; Duvarci & Nader, 2004; Lin et al., 2006), it is inferred that they have been erased, although this is not universally accepted (McGaugh, 2004).

Reports of studies of reconsolidation blockade in animals not infrequently conclude with the suggestion that this mechanism could lead to a novel translational treatment for posttraumatic stress disorder (PTSD). A central feature of PTSD is an overly strong, distressing memory of the causal traumatic event. If these memories could be weakened, substantial suffering might be alleviated. Given that declarative memory and conditioning are mediated by different brain systems and hence are at least partly dissociable, the ideal outcome would be for the patient to retain the declarative ("factual") memory of the traumatic event but lose the associated intense emotion, which has been conceptualized as a conditioned response (CR). Although such a scenario may appear far-fetched, two of the few preclinical human reconsolidation blockade studies, which employed fear conditioning, showed that following the administration of systemic propranolol (a beta-adrenergic blocker that has been reported to block reconsolidation in some animal studies) at the time of memory reactivation, subjects no longer showed the conditioned fear response, but they retained declarative knowledge of the learned contingency (Kindt, Soeter, & Vervliet, 2009; Soeter & Kindt, 2010).

In the only study to date that has attempted to apply reconsolidation blockade to traumatic memories, chronic PTSD patients described their traumatic events, thereby reactivating the memory (Brunet et al., 2008). Shortly afterward, they were given propranolol or placebo. A week later, they engaged in script-driven mental imagery of the event while physiological responses were recorded. Patients who had received postreactivation propranolol showed significantly smaller responses than those who had received placebo, consistent with weakening of the traumatic memory's emotional component. Although this study did not employ sufficient controls to conclude that reconsolidation blockade was the underlying mechanism, it is a viable explanation. One question that emerges from this line of translational research is whether other drugs could possess even stronger reconsolidation-blocking effects and, therefore, be candidates for trials in PTSD either alone or in combination with propranolol. Unfortunately most rat reconsolidation studies employ drugs that either are administered intracerebrally or are too toxic for humans, usually both. Candidate drugs for human use must be capable of safe, systemic administration. It is also desirable that the drug, or drug combination, has been shown to block reconsolidation in animals.

One such candidate drug is the glucocorticoid receptor blocker mifepristone, or RU38486 (most familiar for its use as an abortifacent). Both intraamygdala (Jin et al., 2007) and systemic (Taubenfeld et al., 2009) mifepristone have been shown to block reconsolidation of fear learning in an inhibitory avoidance paradigm in rats. Although inhibitory avoidance may be relevant to PTSD, cue conditioning may be of greater relevance. Psychological distress and physiological reactivity to trauma-related cues have been encoded as *DSM–IV* PTSD criteria B.4 and B.5, respectively.

The present study attempted further to explore in rats the potential of postreactivation mifepristone as a novel treatment for PTSD by testing whether this drug can block reconsolidation of cue-conditioned fear. Additionally, mifepristone was tried with and without concurrently administered propranolol, in order to explore whether the combination of these two drugs would have stronger reconsolidation-blocking effects than either alone. In PTSD, cue and context are usually not so easily separated as they can be in animal research. For example, a Vietnam veteran may be more likely to become distressed at the sight of an Asian male (cue) at night (context). PTSD veterans' fear responses have been found to be excessively augmented by dangerous contexts (Grillon, Morgan, Davis, & Southwick, 1998). For this reason, unlike in many animal studies, the rats underwent conditioning, reactivation, and testing in the same experimental chamber.

Method

Rats

The procedures were approved by the Subcommittee on Research Animal Care (SRAC) of the Massachusetts General Hospital in compliance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Equal numbers of male and female Sprague–Dawley rats (Harlan Laboratories, Indianapolis, IN) weighing ~250g were cohoused (two of the same gender per cage) at the Massachusetts General Hospital Center for Comparative Medicine in transparent polyethylene cages and maintained on a 12-hr light (day)/dark (night) schedule with free access to food and water. They were transported to our laboratory for the study's procedures, which were performed in the early afternoon, and returned to the housing facility at the end of each day. On each of the two days prior to the experiment, rats were handled for five minutes and then placed in the conditioning chamber for five minutes of habituation. Each experimental Plexiglas chamber (Coulburn Instruments, Whitehall, PA) measured $25 \times 29 \times 29$ cm and was situated inside a sound-attenuated box (Med Associates, Burlington, VT).

Drugs

Mifepristone (Sigma, St Louis, MO) in a dose of 7.5 mg (approximately 30 mg/kg) was dissolved in 0.5 ml propylene glycol vehicle. Racemic propranolol (Sigma) in a dose of 2.5 mg (approximately 10 mg/kg) was dissolved in 0.1 ml saline vehicle. Drugs were administered subcutaneously.

Experimental Procedures

On each experimental day, rats were placed in the chamber for 2 min. Then a 4-kHz, 80 dB SPL tone (conditioned stimulus, CS) was presented for 30 sec. Duration of freezing served as the CR and was measured via motion-sensing computer software (FreezeScan, Clever Systems, Reston, VA). Scores are presented as percentage of the total duration of the CS. On Day 1,

rats were trained with a single 1-s 0.75mA shock (US) that was delivered via the grid floor and coterminated with the tone. The rats then remained in the chamber for 1 min and then returned to their home cages. On Day 2 the CS was presented without the US (reactivation). Immediately thereafter the rats were removed from the testing chamber and injected with postreactivation (PR) drug. Drugs were not administered on any other day. However, some rats on Day 2 received nonreactivation (NR) mifepristone without being placed in the chamber. On Days 3 and 10 (one and eight days after reactivation respectively) the CS was again presented without the shock, and the CR was calculated as a measure of PR long-term memory (PR-LTM). Here "long-term" means at least one day following memory reactivation. On Day 11 the US was presented in the absence of the CS (reinstatement). On Day 12, the CS again was presented without the shock, and the CR was calculated as a measure of postreinstatement PR-LTM. There were four PR drug groups: Vehicles alone (VEH), mifepristone (MIF), propranolol (PROP), and both mifepristone and propranolol (MIF + PROP). A fifth group received NR mifepristone (NR_MIF) but did not undergo the reinstatement procedure. A sixth group was tested 4 (instead of 24) hrs after the mifepristone injection in order to measure PR short-term memory (PR-STM). Each of the foregoing groups consisted of 12 male and 12 female rats, with the exception that the reinstatement MIF group comprised only half the original number (i.e., six males and six females).

Data Analysis

The raw dependent measure consisted of percent freezing during each CS presentation, that is, the CR. For testing LTM, percent freezing scores were analyzed by means of a repeated-measures, four-factor analysis of variance (ANOVA) with Gender, MIF (present or absent), and PROP (present or absent) as between-rats effects, and DAY as a repeated measure. LTM after nonreactivated mifepristone, and STM after mifepristone, were analyzed by parallel, three-factor ANOVAs. The experiment-wise alpha of 0.05 (two-tailed) was partitioned in the following manner. There were two major, independent, a priori hypotheses: first that mifepristone would block reconsolidation, and second that mifepristone would interact with propranolol in blocking reconsolidation. For tests subsumed under each of these hypotheses, the threshold for statistical significance was $p < .02$. Given that no study drug was administered on Day 2, interactions with Day were expectable under the a priori hypotheses. For analyses not involving the a priori hypotheses, including all gender main effects and interactions, we divided the remaining alpha of 0.01 by the number of results generated by the four-factor ANOVA unrelated to the two major hypotheses, which was 10, yielding a significance threshold of $p < .001$. For the additional ANOVAs described below, a parallel approach was taken.

Results

Postreactivation Long-Term Memory

Figure 1 displays percent freezing for each group on each test day collapsed across Gender. The four-factor ANOVA on percent freezing scores yielded a significant main effect of gender: $F(1, 112) = 10.7$, $p = .001$; least square means with standard errors in parentheses were: male: 59.6 (2.8), female 46.7 (2.8). However, gender did not significantly interact with any other factor The four-factor ANOVA also yielded a significant DAY x MIF x PROP interaction: $F(3, 112) = 11.5$, $p < .0001$. Stratified by DAY, there was a significant MIF x PROP interaction on Day 3: $F(1, 88) = 5.7$, $p < .02$, and on Day 10: $F(1, 88) = 7.4$, $p = .01$. The MIF x PROP interaction was not significant on Day 2 (when testing was conducted prior the study medication) nor on Day 12 (postreinstatement). Inspection of the Figure 1 Day 3 data indicates the only group that showed attenuated freezing was the MIF group. Stratified by PROP, the mifepristone effect was significant in the absence: $F(1, 44) = 13.2$, $p = .001$, but not in the presence: $F(1, 44) = 0.2$, $p = .64$, of propranolol. The Day 10 data show a similar pattern. Stratified by PROP, the mifepristone effect was again significant in the absence: $F(1, 44) = 13.8$, $p = .001$, but not in the presence: $F(1, 44) = 0.1$, $p = .74$, of propranolol.

Comparison of least square means indicated that freezing in the MIF group decreased from Day 2 to Day 3: $t(88) = 10.9$, $p < .0001$, consistent with blockade of memory reconsolidation. The further (nonsignificant) decrease from Day 3 to Day 10 indicates no spontaneous recovery of the CR. To evaluate whether freezing in the MIF group underwent reinstatement, we tested the difference in least square means between Day 10 (prereinstatement) and Day 12 (postreinstatement), which was significant $t(88) = -2.6$, $p = .01$. However, percent freezing in the MIF group on Day 12 was still significantly lower than it had been on Day 2: $t(88) = 5.5$, $p < .0001$. These results indicate only partial reinstatement of the CR in the MIF group.

Nonreactivation Long-Term Memory

Figure 2 displays mean percent freezing on each test day collapsed across gender in rats that were (PR-MIF) versus were not (NR-MIF) presented with the CS prior to mifepristone. A three-factor ANOVA with GENDER and REACTIVATION as between-rat effects and DAY (Days 3 and 10–Day 2 data are unavailable in nonreactivated rats, and Day 12 reinstatement was not studied) as a repeated measure yielded a main effect of REACTIVATION: $F(1, 44) = 26.2$, $p < .0001$. Inspection of Figure 2 indicates that only when mifepristone was preceded by memory reactivation was there a substantial subsequent decrement in conditioned freezing.

Postreactivation Short-Term Memory

Figure 3 displays percent freezing collapsed across gender following the CS presentation during Day 2 reactivation and again either 4 hrs (PR-STM) or 24 hrs (PR-LTM) later in the MIF group. A three-factor analysis of variance with GENDER and memory TERM (PR-STM or PR-LTM) as between-rat effects and DAY (Days 2 and either Day 2 + 4 hr or Day 3–Days 10 and 12 were not studied) as a repeated measure yielded a main effect of memory TERM: $F(1, 44) = 27.4$, $p < .0001$. Rats in the PR-STM group showed virtually no decrease in freezing.

Discussion

The results of the present study replicate and extend those of an earlier inhibitory avoidance study (Taubenfeld et al., 2009)

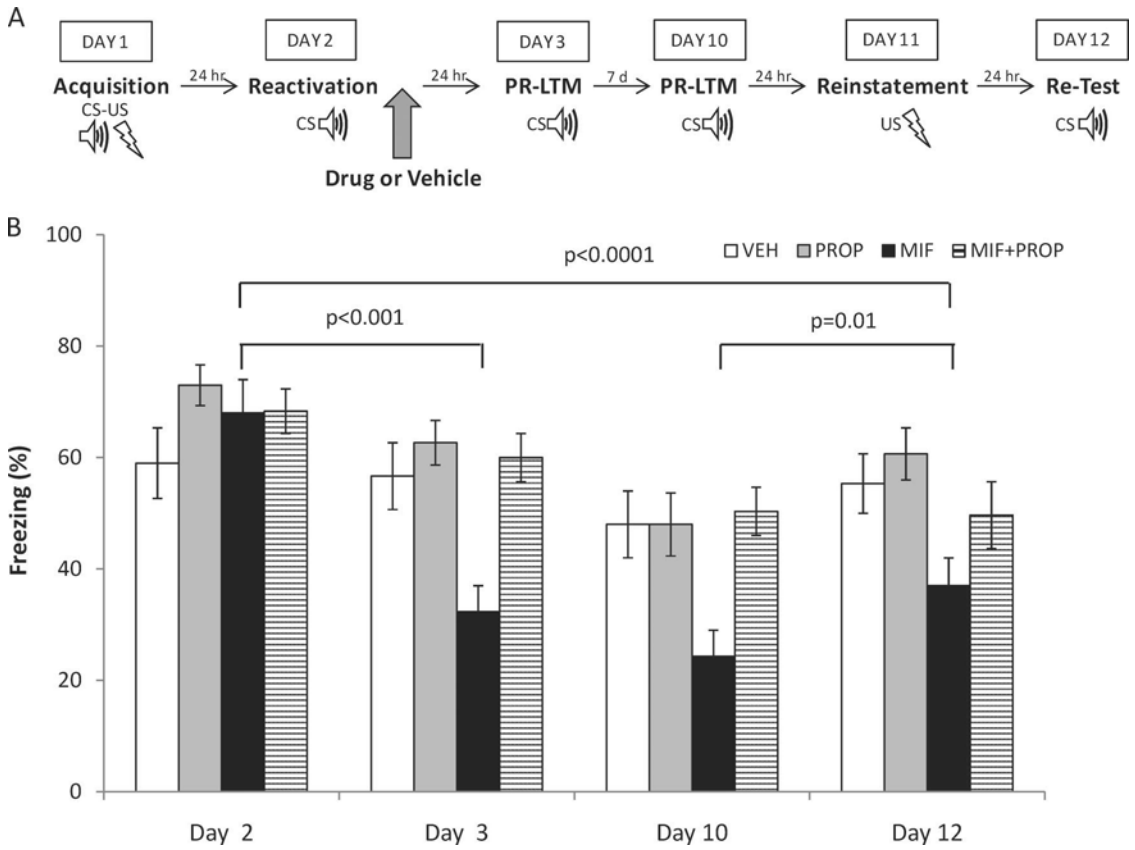

Figure 1. Postreactivation long-term memory (PR-LTM) in the four drug groups. Group mean percentage of freezing to the tone (i.e., conditioned fear response) on Day 2 (reactivation followed by drug), Days 3 and 10 (test days), and Day 12 (test day following reinstatement). See text for details. VEH = vehicle; PROP = propranolol 10 mg/kg; MIF = mifepristone 30 mg/kg; MIF + PROP = both mifepristone and propranolol; Bars = standard error.

by showing that mifepristone administered systemically to rats following the presentation of a previously conditioned fear cue significantly reduced subsequent cue-induced conditioned responding, as manifest in a shorter duration of freezing. The present design incorporated controls necessary to infer that reconsolidation blockade was the mechanism behind this effect. First, the (partial) amnesia for the CS-US association induced by postreactivation mifepristone was relatively long-lasting (for rats), namely, 10 days, that is, there was no evidence of spontaneous recovery. Second, there was only modest reinstatement of the CR in rats that had received mifepristone. Third, nonreactivation mifepristone, that is, drug in the absence of memory reactivation, produced no amnesia. Fourth, when measured four hours following postreactivation mifepristone, the CR was still fully present, whereas it was reduced the next day. Like consolidation, reconsolidation is a time-dependent process that affects long- but not short-term memory.

The present results further suggest that mifepristone is worth exploring in human reconsolidation blockade studies, including as a potential novel treatment for PTSD. A paradoxical result, however, was that concurrent postreactivation propranolol prevented the memory reconsolidation-blocking effect of mifepristone. Propranolol is known to antagonize the memory consolidation-*enhancing* effect of corticosterone by blocking a final common pathway of hormonal modulation of memory, namely, noradrenergic innervation of the basolateral amygdala (Roozendaal et al., 2006). It has been found that basolateral amygdala lesions block not only the memory consolidation-enhancing effect of the glucocorticoid agonist RU28362 (administered intrahippocampally) on inhibitory avoidance, but also the memory consolidation-reducing effect of mifepristone (Roozendaal & McGaugh, 1997). Similar results have been obtained with intraamygdala beta-blockade (Roozendaal B, personal communication of unpublished data). The present results extend these findings to reconsolidation, in that we found that systemic propranolol blocked the reconsolidation-*reducing* effect of mifepristone. This finding suggests that a permissive level of (nor)adrenergic activity is required not only for the memory-enhancing effects of glucocorticoids but also for the memory-reducing effects of their antagonists. The mechanism of this permission remains to be elucidated. From a translational standpoint, the finding that propranolol prevents rather than enhances the reconsolidation-blocking effect of mifepristone, at least in the doses used here, militates against attempting to combine these two drugs in a reconsolidation-blockade treatment approach to PTSD.

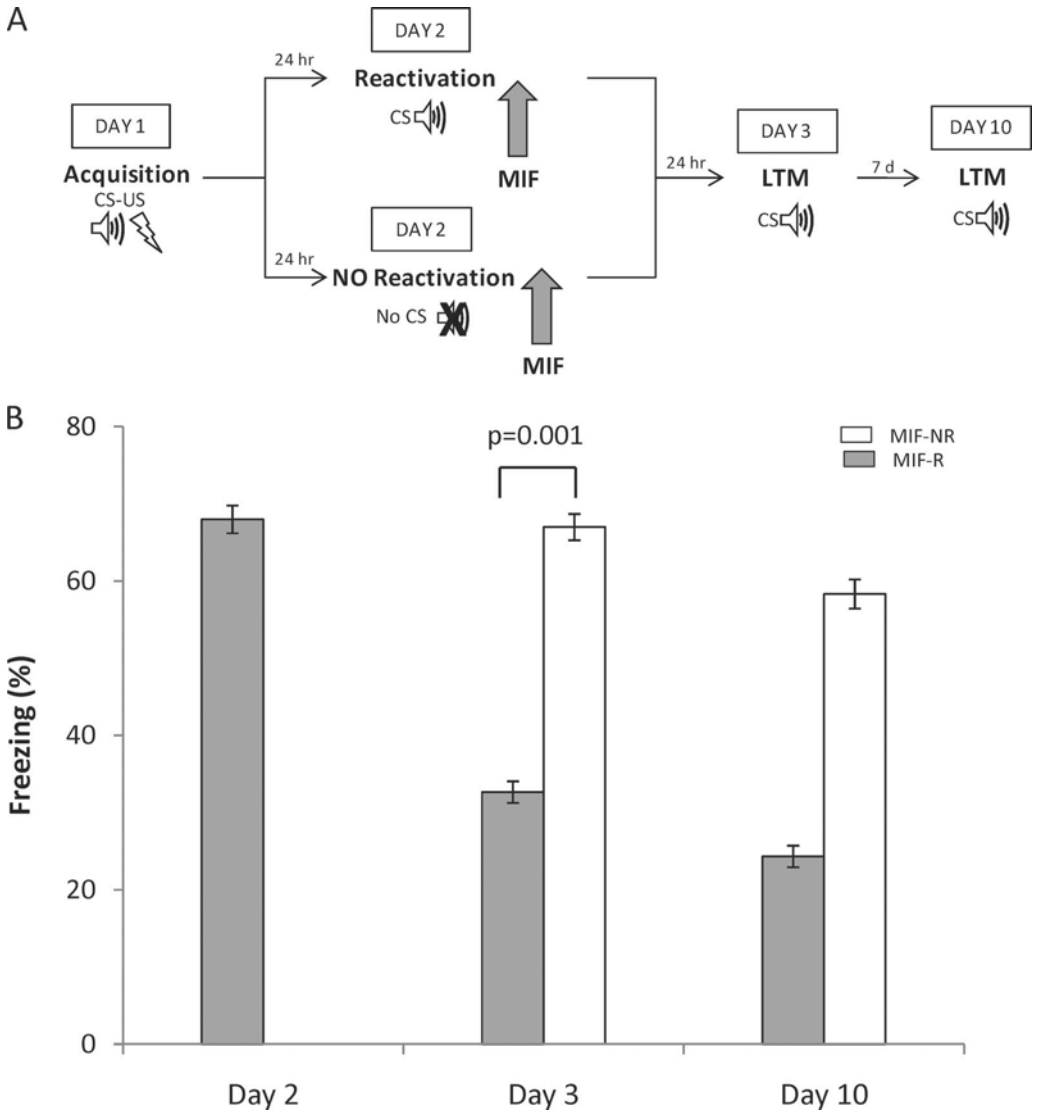

Figure 2. Postreactivation long-term memory (PR-LTM) in the nonreactivated versus reactivated mifepristone groups. Group mean percentage of freezing (i.e., conditioned fear response) on Day 2 (mifepristone preceded or not preceded by reactivation) and Days 3 and 10 (test days). MIF = mifepristone 30 mg/kg; NR = nonreactivation; R = reactivation. No Day 2 data are shown for the NR group because the conditioned stimulus was not presented to this group on that day. Bars = standard error.

In the present study, systemic postreactivation propranolol alone did not block reconsolidation of conditioned fear. This negative result is partially at odds with results of some previously published studies that used the same 10 mg/kg dose as in the present study (Debiec & Ledoux, 2004; Muravieva & Alberini, 2010; Przybyslawski et al., 1999) or nearly the same dose (5 mg/kg; Abrari et al., 2008). The discrepancy might be explained by design and methodological differences. The present study used a cue-conditioning procedure whereas one of these previous positive studies employed inhibitory avoidance (Przybyslawski et al., 1999) and one employed context conditioning (Abrari et al., 2008). Of the two studies reporting that propranolol blocked reconsolidation of cue conditioning, one (Muravieva & Alberini, 2010) used Long Evans, rather than Sprague–Dawley rats as herein. In both cue-conditioning studies (Debiec & Ledoux, 2004; Muravieva & Alberini, 2010), the conditioned responses were acquired in one experimental chamber (context), but reactivated and then tested in another chamber. For reasons of clinical applicability described above, in the present study all procedures were performed in the same chamber.

Interestingly, in the last of the two above studies (Muravieva & Alberini, 2010), propranolol failed to block the reconsolidation of inhibitory avoidance, whereas systemic mifepristone had previously succeeded in doing so in a study in the same laboratory (Taubenfeld et al., 2009). In addition to the present results, this suggests that, compared to propranolol, mifepristone may be a superior reconsolidation blocker of conditioned fear across various designs and may ultimately turn out to be a more useful treatment

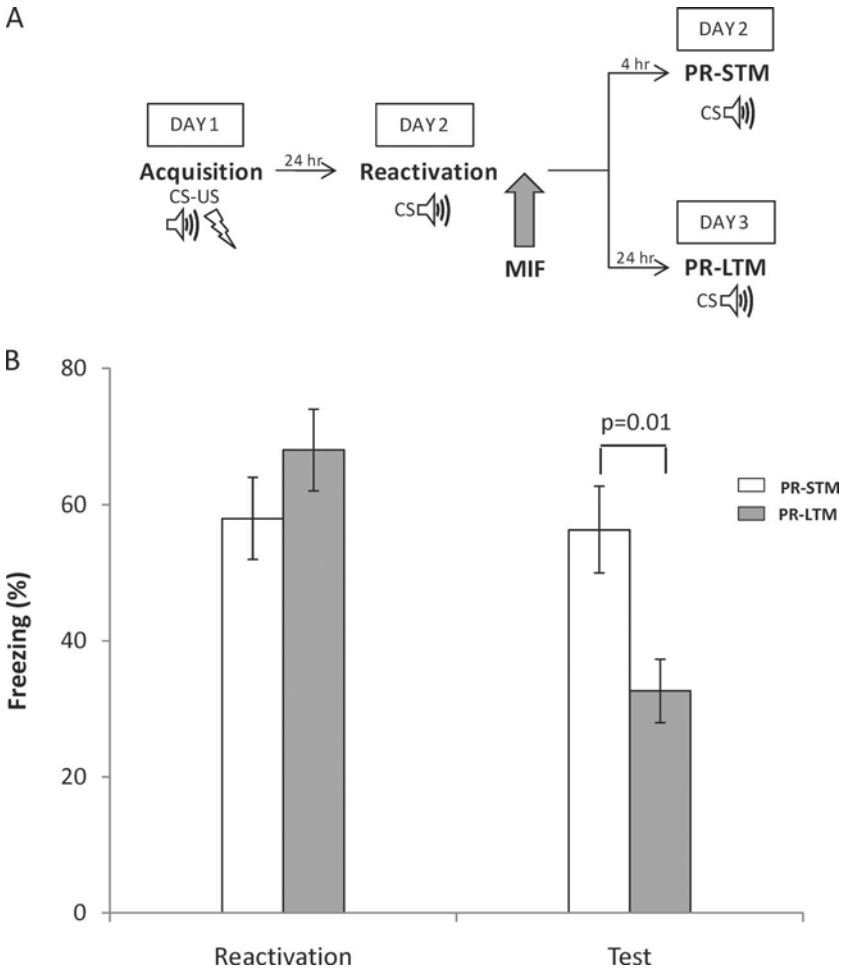

Figure 3. Postreactivation short-term memory (PR-STM) versus postreactivation long-term memory (PR-LTM) in mifepristone groups. Group mean percentage of freezing (i.e., conditioned fear response) on Day 2 (reactivation followed by mifepristone 30 mg/kg) and again either 4 (MIF_PR-STM) or 24 (MIF_PR-LTM) hrs later. Bars = standard error.

for PTSD. At any rate, results of translational studies in animals can only identify effects that deserve further investigation in humans; one-to-one correspondence is not assured.

This study has several limitations. For reasons discussed in the introduction, CRs were only tested in a single context (chamber). Consequently, renewal could not be assessed. Due to the lack of a quantification of freezing to the context prior to the CS presentation, the possibility that context conditioning played some role in the observed results cannot be ruled out. The present design employed only single doses of mifepristone (30 mg/kg) and propranolol (10 mg/kg). These doses were chosen on the basis of their having most often been used in relevant published rat studies, and the consideration that higher doses on a translational mg/kg basis could be prohibitive in humans. The possibilities that different doses of each drug might produce greater reconsolidation blockade, and that different doses of the two drugs in combination might allow mifepristone to block reconsolidation cannot be ruled out.

It could be that the mifepristone-propranolol interaction observed in the present study was pharmacokinetic rather than pharmacodynamic in nature. In other words, one of the drugs may have increased or decreased metabolism of the other, thereby affecting blood levels. However, this explanation is unlikely given that such a pharmacokinetic interaction has not been previously reported and that the metabolism of mifepristone and propranolol rely upon different cytochrome P450 enzymes (Jang, Wrighton, & Benet, 1996; Yoshimoto, Echizen, Chiba, Tani, & Ishizaki, 1995). The relatively high dose of propranolol used here could have caused effects other beta-adrenergic (e.g., serotonergic). Although the mifepristone results have been interpreted within the framework of glucocorticoid receptor blockade, this drug has other, especially antiprogesterone, properties which could partially underlie its observed effect. Because mifepristone is currently the only suitable glucocorticoid receptor blocker approved for human use, this limitation was unavoidable. Although the underlying mechanism of action is of scientific interest, the nature of this action may not be of great concern from a clinical standpoint. The primary objective of the present study was to test

reconsolidation-blockers as potential candidates for treating PTSD, regardless of their mechanisms of action.

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Pharmacological blockade of memory reconsolidation in posttraumatic stress disorder: Three negative psychophysiological studies

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ABSTRACT

Posttraumatic stress disorder (PTSD) may involve over-consolidated emotional memories of the traumatic event. Reactivation (RP) can return a memory to an unstable state, from which it must be restabilized (reconsolidated) if it is to persist. Pharmacological agents administered while the memory is unstable have been shown to impair reconsolidation. The N-methyl-D-aspartate (NMDA) partial agonist D-cycloserine (DCS) may promote memory destabilization. In the three studies reported here, we investigated whether the β-adrenergic blocker propranolol or the glucocorticoid (GR) antagonist mifepristone, given at the time of traumatic memory reactivation, could reduce PTSD symptoms and physiological responding during subsequent traumatic imagery. Individuals with PTSD were randomized as follows: Study One: propranolol with memory reactivation ($n=10$) or without reactivation ($n=8$); Study Two: reactivation mifepristone ($n=13$), non-reactivation (NRP) mifepristone ($n=15$), or double placebo (PL) ($n=15$); Study Three: reactivation mifepristone plus D-cycloserine ($n=16$), or two placebos ($n=15$). Subjects underwent memory retrieval by describing their traumatic event. A week later they engaged in script-driven traumatic mental imagery, while heart rate (HR), skin conductance (SC), and facial electromyogram (EMG) responses were measured. There were no significant group differences in physiological responsivity or change in PTSD symptoms in any of the studies. These results do not support successful blockade of reconsolidation of traumatic memories in PTSD.

1. General introduction

Animal research suggests that under favorable conditions, the retrieval (reactivation (RP)) of a consolidated memory may return it to a labile state from which it must be restabilized in order to persist (Nader et al., 2000). This restabilization process is termed reconsolidation. It involves neurobiological mechanisms that are similar but not identical to those involved in memory consolidation (Lee et al., 2004). Reconsolidation is largely demonstrated by its blockade. It derives its support from experiments in many species including humans, and a variety of experimental paradigms using a broad range of interventions, including localized or systemic drug administration (Nader and Einarsson, 2010; Debiec and Ledoux, 2004). Pharmacological reconsolidation blockade is a two-stage process. First, the memory must be destabilized by reactivating (retrieving) it. Only destabilized memories are able to undergo modification or blockade. Second, reconsolidation of the memory must be antagonized by a pharmacological agent. Reactivated fear memories have been shown to be sensitive to β-adrenergic blockers such as propranolol in animals (Przybyslawski et al., 1999; Debiec and Ledoux, 2004) and in humans (Kindt et al., 2009; Soeter and Kindt, 2010), and to glucocorticoid (GR) antagonists such as mifepristone (RU-486) in animals (Jin et al., 2007; Taubenfeld et al., 2009; Pitman et al., 2011). Many articles about reconsolidation blockade conclude with the suggestion that it could offer a novel treatment for posttraumatic stress disorder (PTSD), which is characterized by durable, distressing emotional memories. Administering a suitable

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drug during retrieval-induced destabilization might reduce the strength of a traumatic memory by blocking its reconsolidation.

In a preliminary, placebo (PL)-controlled, clinical investigation of pharmacological reconsolidation blockade, Brunet et al. (2008) employed a validated psychophysiological script-driven imagery technique in 19 subjects with PTSD resulting from various traumatic events. In previous studies, physiological responses during traumatic imagery had been shown to reliably discriminate trauma-exposed individuals with PTSD from trauma-exposed individuals without PTSD (Orr et al., 2002). Subjects in the Brunet et al. study underwent a script preparation procedure that entailed their describing their traumatic event, which hypothetically served to reactivate the traumatic memory. Immediately afterwards they received propranolol or placebo. A week later, they engaged in script-driven mental imagery of their traumatic event, while heart rate (HR), skin conductance (SC), and left corrugator electromyogram (EMG) were measured. In comparison to subjects who had received placebo, overall physiological responding during mental imagery of the traumatic event was significantly smaller in the subjects who had received post-reactivation propranolol a week earlier, suggesting that the traumatic memory had been weakened. The objectives of the three studies reported here were to expand upon this previous study and to investigate new pharmacological agents as potential reconsolidation blockers in PTSD.

2. Study One

2.1. Introduction

One limitation of the Brunet et al. (2008) study was that it did not include a non-reactivation (NRP) propranolol group; consequently, the possibility that non-specific actions of propranolol were responsible for the effect could not be ruled out. Study One therefore had two aims: first, further to investigate whether propranolol administered with memory reactivation weakens traumatic memories associated with PTSD; and second, to rule out the possibility that such an effect, if found, is due to non-specific actions of this drug. We hypothesized that individuals with PTSD who underwent memory reactivation via script preparation accompanied by propranolol (reactivation, RP) would show smaller physiological responses during script-driven imagery testing a week later compared to those who received propranolol in the absence of the script preparation procedure (non-reactivation, NRP).

2.2. Methods

2.2.1. Subjects

2.2.1.1. Recruitment and inclusion criteria. Subject candidates were male veterans ages 24–64 who had received a clinical diagnosis of combat-related PTSD (American Psychiatric Association, 2000). They were drawn from referrals from the VA Medical Centers in Bedford, MA and Manchester, NH, as well as from advertisements in the media.

2.2.1.2. Exclusion criteria. Prior to enrollment, subject candidates were clinically screened and excluded if they had a history of a psychotic or bipolar I disorder; a current substance use disorder; a medical condition that contraindicated the administration of propranolol, e.g., congestive heart failure, diabetes, chronic bronchitis, or emphysema; a history of an asthmatic attack within the past 10 years, a history of an asthmatic attack precipitated by a β-adrenergic blocker at any time in the past, or currently being treated for asthma regardless of when the last attack occurred; previous adverse reaction to, or non-compliance with, a β-adrenergic blocker; initiation of, or change in, psychotropic medication within 1 month prior to recruitment; current use of a medication that may have dangerous interactions with propranolol, e.g., other β-adrenergic blockers, antiarrhythmics, and calcium channel blockers; resting heart rate <60 beats per minute or resting systolic blood pressure <100 mm Hg.

2.2.1.3. Ethical approval and informed consent. After a complete explanation of the study procedures, which had been approved by the Partners Human Research Committee, the Manchester/Bedford VA Medical Centers Human Studies Subcommittee, and the U.S. Army Medical Research and Materiel Command Human Research Protection Office, subjects gave written informed consent for participation.

2.2.2. Study medication

A double-blind 1:1 randomization schedule was utilized. Propranolol hydrochloride is a lipophilic, non-selective synthetic β_1- and β_2-adrenoreceptor antagonist that crosses the blood brain barrier. On Day 0 and Day 2, we administered either a first dose of 0.67 mg/kg short-acting (SA) oral propranolol (rounded to the nearest 10 mg) or matching placebo. If the SA dose was well-tolerated (which it was in all subjects), and if systolic blood pressure had not decreased by more than 10 mm Hg to below a level of 100 mm Hg (which did not happen in any subject), 90 min later (and immediately prior to script preparation), either 1 mg/kg of long-acting (LA) oral propranolol (rounded to the nearest 20 mg) or placebo was also administered. Subjects were given the SA propranolol 90 min prior to memory retrieval in order to allow the drug to have reached an adequate plasma concentration at the time of traumatic memory reactivation. The study medication was well tolerated by all subjects.

2.2.3. Equipment and physiological measures

A Coulbourn Lablinc V Human Measurement System (Coulbourn Instruments, Allentown, Pennsylvania) was used to record physiological analog signals, including heart rate (HR), skin conductance (SC), and electromyogram (EMG) of the left corrugator and left lateral frontalis facial muscles. Interbeat interval was recorded via standard limb electrocardiogram leads connected to a High Gain Bioamplifier (V75-04) and converted to HR. SC was measured by a Coulbourn Isolated Skin Conductance coupler (V71-23) using a constant 0.5 V through 8 mm (sensor diameter) Invivo Metric Ag/AgCl electrodes placed on the hypothenar surface of the subject's non-dominant hand in accordance with published guidelines (Fowles et al., 1981). The SC electrodes were separated by 14 mm, as determined by the width of the adhesive collar. For EMG recordings, the skin was lightly abraded, and 4 mm (sensor diameter) Invivo Metric Ag/AgCl electrodes filled with electrolyte paste were placed over the corrugator and frontalis muscle sites according to published specifications (Fridlund and Cacioppo, 1986). The EMG was amplified by a Coulbourn High Gain Bioamplifier (V75-04), filtered so as to retain the 90–1000 Hz frequency range, and integrated by a Coulbourn Contour Following Integrator (V76-23A) with a 200 ms time constant. Physiological analog signals were digitized by a Coulbourn analog to digital converter (V19-16). A Cobalt notebook computer (IBM-compatible) with custom-designed software was used to sample and store the digitized physiological signals.

2.2.4. Procedures

On Day 0 (non-reactivation), subjects randomized to the NRP group received SA and LA propranolol, whereas subjects randomized to the RP group received matching placebo capsules. All subjects then viewed a 90 min emotionally neutral movie. By

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design, subjects were not permitted to discuss their combat events or PTSD symptoms on Day 0 to reduce the chances of inadvertent traumatic memory reactivation.

On Day 2 (reactivation), subjects in the RP group received SA and LA propranolol, whereas subjects in the NRP group received placebos. 90 min after the SA dose, all subjects underwent a script-preparation session as previously described (Pitman et al., 1987). In brief, subjects recalled and provided written details of two traumatic experiences, or two aspects of the same traumatic experience, which had led to their PTSD, as well as three other personal (non-traumatic) life experiences. They then selected bodily responses that accompanied each experience. An investigator later composed approximately 30-s scripts portraying each experience and incorporating up to five of the selected bodily responses. Subjects also completed a baseline Impact of Event Scale-Revised (IES-R; Weiss and Marmar, 1997) for each of their five personal events; however, only the IES-R scores for the two traumatic scripts were subjected to analysis. During the 90-min period prior to script preparation and continuing afterwards if necessary, a doctoral-level psychologist administered the Clinician-Administered PTSD Scale: Current and Lifetime Diagnosis Version (CAPS-DX; Blake et al., 1995) to verify the presence of current, combat-related PTSD, and the Structured Clinical Interview for DSM-IV (SCID; First et al., 2007) to evaluate the presence of any other Axis I comorbidity. The CAPS and SCID were administered on Day 2 in order to reduce the chances of inadvertent traumatic memory reactivation on Day 0.

On Day 8 (i.e., approximately 1 week later), urine samples were collected and sent for analysis of substances of abuse. Subjects then underwent the script-driven imagery testing session as previously described (Pitman et al., 1987). In brief, physiological recording electrodes were placed on the subject's face and arms. The subject then listened to 11 scripts presented sequentially in pseudorandom order, consisting of the five personal scripts prepared on Day 2 and six standard scripts. Each script presentation consisted of four sequential 30-s periods: baseline, listening, imagery, and recovery during each of which physiological measures were recorded. Following the script-driven imagery procedure, subjects again completed IES-R scores for each of the five personal events that they had described on Day 2.

2.2.5. Data reduction and statistical analysis

Response scores for each physiological measure for each script were calculated by subtracting the 30-s baseline period mean from the 30-s imagery period mean. Responses to the two traumatic scripts were averaged and square-root transformed prior to analysis. An a priori discriminant function was derived from the HR, SC, and lateral frontalis EMG responses of 92 individuals with PTSD and 86 individuals without PTSD, who had previously been studied using the same script-driven imagery technique. This discriminant function was used to a) derive PTSD cut-off scores (shown in Tables 1–3) and b) calculate each subject's probability of being classified into the physiological PTSD group (Orr et al., 2012; Bauer et al., 2013; Pineles et al., 2013). This physiological PTSD probability score (PPrb) served as a composite measure of overall physiological responding during script-driven traumatic imagery, obviating the need for multivariate analyses of physiological responses in the small samples studied. In cases for which one of the three physiological measures was missing due to technical failure, PPrb was calculated on the basis of the remaining two measures. IES-R change scores were calculated for each of the two traumatic combat scripts by subtracting the Day 2 IES-R score from the Day 8 IES-R score. Raw IES-R scores at Days 2 and 8, and IES-R change scores from Day 2 to Day 8 were averaged for the two traumatic scripts.

Between-group Student's *t*-tests were performed for all outcome measures. The threshold for statistical significance was $p < 0.05$ (two-tailed), except for each individual physiological response measure, where the Bonferroni-corrected significance threshold was $p < 0.0125$ (i.e. $0.05 \div 4$ physiological measures). For the outcome measures of primary interest, viz., PPrb score and Day 2 to Day 8 IES-R change score, 95% confidence intervals were calculated.

2.3. Results

2.3.1. Subject randomization and characteristics

One subject who was randomized to the RP group was withdrawn from the study following his relapse into opioid abuse after his Day 2 participation. One subject also randomized to RP, and one subject randomized to NRP, dropped out following their Day 2 participation. Two subjects randomized to NRP did not meet current PTSD diagnostic criteria as determined by the CAPS on Day 2. Data from these five subjects were excluded from the analysis, leaving final group sizes of RP $n=10$ and NRP $n=8$.

As shown in the top panel of Table 1, there were no significant group differences in age, baseline IES-R score, or CAPS score. Current comorbid mental disorders according to the SCID included, in the RP group: major depressive disorder (MDD, $n=2$), panic disorder ($n=2$), simple phobia ($n=2$), social phobia

Table 1
Study One demographics, psychometrics, and psychophysiological responses.

	NR propranolol $n=8$ (all male)	**R propranolol** $n=10$ (all male)	d.f.	*t*	*p*
Baseline measures					
Age	33.3 (11.5)	38.7 (14.9)	16	0.85	0.41
Day 2 IES-R score	43.3 (14.2)	45.0 (18.3)	16	0.22	0.83
Clinician Admin PTSD Scale	58.6 (14.8)	62.7 (13.7)	16	0.61	0.55
Outcome measures[a]					
Day 8 IES-R score	34.3 (15.8)	51.8 (16.4)	13[b]	2.06	0.06
Change in IES-R score	−8.2 (13.0)	4.5 (13.2)	13[b]	1.83	0.09
Physiological PTSD probability score	0.32 (0.11)	0.45 (0.21)	15[c]	1.45	0.17
Heart rate response ($\sqrt{BPM}$) (empirical PTSD cut-off = 1.9)	0.82 (1.08)	0.76 (1.78)	15[c]	−0.09	0.93
Skin conductance response ($\sqrt{\mu S}$) (empirical PTSD cut-off = 0.5)	0.19 (0.85)	0.42 (0.64)	15[c]	0.64	0.53
Frontalis EMG response ($\sqrt{\mu V}$) (empirical PTSD cut-off = 1.1)	0.01 (0.66)	0.65 (0.77)	15[c]	1.78	0.10
Corrugator EMG response ($\sqrt{\mu V}$) (empirical PTSD cut-off = 1.5)	0.54 (0.64)	0.79 (1.37)	15	0.04	0.67

NR = non-reactivation, R = reactivation; CI = confidence interval; IES-R: Impact of Event Scale-Revised; PTSD = posttraumatic stress disorder; BPM = beats per minute; μS = microsiemens; μV = microvolts.

[a] All physiological outcome measures are square-root transformed.
[b] Data missing in three subjects.
[c] Data missing in one subject.

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Table 2
Study Two demographics, psychometrics, and psychophysiological responses.

	Placebo – Placebo $n=15$ (two female)	**NR mifepristone** $n=15$ (five female)	**R mifepristone** $n=13$ (three female)	d.f.	*F*	*p*
Baseline measures						
Age	40.5 (11.7)	44.7 (10.4)	46.8 (14.5)	2.40	0.99	0.38
Day 2 IES-R score	59.5 (15.8)	47.9 (18.2)	46.3 (20.6)	2.40	2.27	0.12
Clinician Admin PTSD Scale	62.5 (17.9)	59.1 (9.9)	57.3 (14.4)	2.40	0.48	0.62
Outcome measures[a]						
Day 8 IES-R score	49.4 (23.1)	47.3 (19.5)	40.4 (18.5)	2.40	0.72	0.50
Change in IES-R score	−10.1 (16.9)	−0.6 (9.6)	−5.9 (14.2)	2.40	1.76	0.19
Physiological PTSD probability score	0.40 (0.23)	0.43 (0.17)	0.40 (0.23)	2.40	0.10	0.90
Heart rate response ($\sqrt{BPM}$) (empirical PTSD cut-off=1.9)	0.78 (2.01)	1.52 (1.10)	0.60 (1.50)	2.40	1.38	0.26
Skin conductance response ($\sqrt{\mu S}$) (empirical PTSD cut-off=0.5)	−0.27 (2.20)	1.62 (2.88)	0.43 (0.46)	2.34[b]	2.52	0.10
Frontalis EMG response ($\sqrt{\mu V}$) (empirical PTSD cut-off=1.1)	0.81 (1.07)	0.50 (1.12)	0.81 (1.44)	2.40	0.32	0.73
Corrugator EMG response ($\sqrt{\mu V}$) (empirical PTSD cut-off=1.5)	1.35 (1.44)	1.48 (1.19)	1.89 (1.96)	2.38[c]	0.43	0.65

R=reactivation; IES-R=Impact of Event Scale-Revised; PTSD=posttraumatic stress disorder; BPM=beats per minute; μS=microsiemens; μV=microvolts.

[a] All physiological outcome measures are square-root transformed.
[b] Data missing in six subjects.
[c] Data missing in two subjects.

Table 3
Study Three demographics, psychometrics, and psychophysiological responses.

	Placebo+Placebo $n=15$ (eight female)	**Mifepristone+DCS** $n=16$ (nine female)	d.f.	*t*	*p*
Baseline measures					
Age	35.1 (11.8)	41.9 (13.9)	29	1.48	0.15
Day 7 IES-R score	55.3 (21.9)	52.4 (15.3)	29	−0.43	0.67
Clinician Admin PTSD Scale	61.6 (17.5)	66.9 (10.4)	29	1.04	0.31
Outcome measures[a]					
Day 14 IES-R score	50.3 (28.2)	41.6 (18.0)	28[b]	−1.01	0.32
Change in IES-R score	−5.0 (16.6)	−8.9 (11.9)	28[b]	−0.75	0.46
Physiological PTSD probability score	0.44 (0.24)	0.45 (0.22)	29	0.05	0.96
Heart rate response ($\sqrt{BPM}$) (empirical PTSD cut-off=1.9)	0.96 (1.97)	1.49 (0.95)	29	0.97	0.34
Skin conductance response ($\sqrt{\mu S}$) (empirical PTSD cut-off=0.5)	0.58 (0.67)	0.47 (0.75)	21[c]	−0.36	0.73
Frontalis EMG response ($\sqrt{\mu V}$) (empirical PTSD cut-off=1.1)	0.59 (1.13)	0.70 (0.93)	29	0.31	0.76
Corrugator EMG response ($\sqrt{\mu V}$) (empirical PTSD cut-off=1.5)	1.25 (1.77)	1.45 (1.24)	29	0.37	0.72

R=reactivation; IES-R=Impact of Event Scale-Revised; PTSD=posttraumatic stress disorder; BPM=beats per minute; μS=microsiemens; μV=microvolts.

[a] All physiological outcome measures are square-root transformed.
[b] Data missing in one subject.
[c] Data missing in eight subjects.

($n=2$), bipolar II ($n=1$), generalized anxiety disorder (GAD, $n=1$); in the NRP group: MDD ($n=4$), panic disorder ($n=1$), social phobia ($n=1$), obsessive–compulsive disorder (OCD, $n=1$).

2.3.2. Outcome measures

As shown in Table 1, the group difference in Day 8 PPrb score, a measure of overall physiological reactivity during script-driven traumatic imagery, was not significant. Specifically, the observed difference in group means was −0.13 (effect size Hedge's $g=-0.71$), which was in the *non*-predicted direction (higher mean in the reactivation propranolol than in the nonreactivation propranolol group). The 95% confidence interval for the group mean difference was −0.30 to 0.04 (effect size confidence interval $g=-1.67$ to 0.24). There were also no significant group differences on any individual physiological response measure, on Day 2 or Day 8 IER-S scores, or on IES-R change score. For IES-R change score, the observed difference in group means was −12.7 (effect size $g=-0.91$), which was in the *non*-predicted direction (lesser decline from Day 2 to Day 8 in the reactivation propranolol group than in the nonreactivation propranolol group). The 95% confidence interval for the group mean difference was −27.4 to 1.9 (effect size confidence interval $g=-1.98$ to 0.15). Note that the preceding small confidence limits in the predicted direction for PPrb and IES-R change score suggest that failure to find the hypothesized effect of reactivation propranolol did not represent a Type II error.

According to urine testing on Day 8, four subjects were found to be taking one or more potentially confounding substances, including opiates, barbiturates, and methadone, at the time of the script-driven imagery procedure. When the analyses were repeated excluding these subjects, the group difference in physiological responses remained non-significant. There were significant group differences in Day 8 IES-R score (NRP $n=5$: $M=29.7$, S.D.=12.5; RP $n=6$: $M=54.3$, S.D.=18.5; $t(9)=2.5$, $p=0.03$) and IES-R change scores (NRP $n=5$: $M=-10.2$, S.D.=13.4; RP $n=6$: $M=11.4$, S.D.=9.8; $t(9)=3.1$, $p=0.01$; IES data were missing in three subjects). However these results should be regarded with caution because of the small sample sizes and non-predicted direction of the group difference.

2.4. Discussion

The results of Study One failed to replicate the previous findings of Brunet et al. (2008), which had suggested that traumatic memory

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reactivation plus propranolol reduces physiological responding during subsequent traumatic mental imagery. However, the individual physiological responses of the NRP control group in Study One were only slightly higher than those exhibited by the post-reactivation propranolol group in the study of Brunet et al. Moreover, the NRP control group's PPrb was below the cut-off for PTSD (0.50). It is possible that non-specific effects of propranolol (i.e., effects unrelated to traumatic memory reactivation) could have lowered the physiological responses in both groups a week later. Had we included a third group that received placebo on both Day 0 and Day 2, and had such a group shown the high physiological responses expected of persons with PTSD, such an interpretation might have been supported.

Cohort demographics also differed between the present study and that of Brunet et al. (2008). Specifically, the present study recruited only male subjects with combat-related PTSD, whereas the earlier study had included both men and women with a range of causal traumatic events. A *post-hoc* analysis of the earlier study's data revealed a greater effect of post-reactivation propranolol in the female subjects (Brunet, unpublished results). Finally, the Brunet et al. (2008) and the present study differed in that the former employed post-reactivation propranolol administration, whereas the present study, for reasons discussed above, employed pre-reactivation propranolol.

3. Study Two

3.1. Introduction

In this study, we considered another pharmacological agent that has offered promise as a reconsolidation blocker. Mifepristone (RU-486) is widely and safely used as an abortifacient due to its anti-progestin effects, but it is also a powerful GR antagonist. We hypothesized that individuals with PTSD whose traumatic memories putatively underwent reactivation via script preparation that was accompanied by mifepristone (reactivation, RM) would show smaller physiological responses during script-driven imagery testing a week later compared to those who received either mifepristone in the absence of the script preparation procedure (non-reactivation, NRM) or double-placebo controls (PP).

3.2. Methods

3.2.1. Subjects

3.2.1.1. Recruitment and inclusion criteria. Research subjects were males and females ages 18–73 who met diagnostic criteria for PTSD. They were drawn from advertisements in the media in the Boston area and referrals at a large southwestern VA Medical Center.

3.2.1.2. Exclusion criteria. Prior to enrollment, subject candidates were clinically screened and excluded if they had a history of psychotic or bipolar I disorder or current substance use disorder. Additional exclusion criteria included: medical condition that contra-indicated the administration of mifepristone such as history of adrenal failure; concurrent corticosteroid or anticoagulant therapy; hemorrhagic disorder; cardiovascular, hypertensive, hepatic, respiratory or renal disease; insulin dependent diabetes mellitus; severe anemia; heavy smoking; porphyria, allergy to mifepristone; pregnant or currently breastfeeding; initiation of, or change in, psychotropic medication within 1 month prior to recruitment; and current use of medication that may involve potentially dangerous interactions with mifepristone, including certain CYP 3A4 substrates such as calcium channel blockers, azole antifungals, macrolide antibiotics, and tricyclic antidepressants.

3.2.1.3. Ethical approval and informed consent. An investigational new drug number (IND) for the off-label use of mifepristone in Study Two and in Study Three (below) was obtained from the U.S. Food and Drug Administration (FDA). After a complete explanation of the study procedures, which had been approved by the Partners Human Research Committee, VA North Texas Health Care System Institutional Review Board, and the U.S. Army Medical Research and Materiel Command Human Research Protection Office, subjects gave written informed consent for participation.

3.2.2. Study medication

Mifepristone (Danco Laboratories, LLC, New York, NY) is a synthetic steroid that acts as a glucocorticoid and progesterone receptor antagonist and as a weak antiandrogen. Following oral administration, mifepristone reaches a peak plasma concentration after approximately 90 min. In a pre-clinical animal study, reconsolidation blockade was found with a dose of 30 mg/kg, which on a kg-for-kg basis corresponds to approximately 1800 mg in a 60 kg human (Pitman et al., 2011). The 1800 mg mifepristone dose employed here represents the maximum that has been approved for use in the USA (albeit for a different indication). On each of Day 0 and Day 2, we administered either 1800 mg oral mifepristone or placebo. Subjects randomized to the RM group received placebo on Day 0 (non-reactivation) and mifepristone on Day 2 (reactivation). Subjects randomized to the NRM group received mifepristone on Day 0 and placebo on Day 2. Subjects randomized to the PP group received placebo on Day 0 and again on Day 2. Thus, both the NRM and PP groups underwent reactivation via script preparation in the absence of mifepristone. A double-blind, 1:1:1 randomization schedule was utilized. The study medication was well tolerated by all subjects with few reported side effects.

3.2.3. Equipment and physiological measures

See Section 2.

3.2.4. Procedures

These were as in Study One, with the addition that female subjects of child-bearing potential underwent a serum pregnancy test on Day 0 prior to receiving study medication. A positive pregnancy test would have resulted in exclusion from the remainder of the study (all participants tested negative).

3.2.5. Data reduction and statistical analysis

These were as in Study One, except that one-way analyses of variance (ANOVAs) with three levels (RM, NRM, PP) were performed in place of *t*-tests. Two-way ANOVA was also performed to incorporate gender into the analyses.

3.3. Results

3.3.1. Subject randomization and characteristics

Six subjects randomized to RM, two subjects randomized to NRM, and two subjects randomized to PP did not meet PTSD diagnostic criteria as determined by the CAPS on Day 2. Data from these subjects were excluded from the analysis, leaving final group sizes of RM $n=13$ (three female), NRM $n=15$ (five female), and PP $n=15$ (two female).

As shown in the top panel of Table 2, there were no significant group differences in age, baseline IES-R score, or CAPS score. Current comorbid mental disorders according to the SCID included, in the RM group: MDD ($n=3$), panic disorder ($n=3$), bipolar II ($n=1$), dysthymia ($n=1$), simple phobia ($n=1$); in the NRM group: MDD ($n=7$), social phobia ($n=3$), simple phobia ($n=3$), bipolar II ($n=1$), dysthymia ($n=1$), eating disorder ($n=1$), GAD ($n=1$), OCD ($n=1$), pain disorder ($n=1$); and in the PP group: MDD ($n=3$), social phobia

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($n=2$), bipolar II ($n=1$), eating disorder ($n=1$), GAD ($n=1$). Traumatic events were as follows: in the RM group: combat ($n=2$), actual or threatened serious injury to self ($n=6$), rape or sexual violence ($n=3$), witnessing death or serious injury of others ($n=2$); in the NRM group: combat ($n=4$), actual or threatened serious injury to self ($n=2$), rape or sexual violence ($n=2$), witnessing death or serious injury of others ($n=7$); PP group: combat ($n=5$), actual or threatened serious injury to self ($n=3$), rape or sexual violence ($n=5$), witnessing death or serious injury of others ($n=2$).

3.3.2. Outcome measures

As shown in Table 2, there were no significant group differences in Day 8 PPrb score. Specifically, the observed difference in group means between the RM and PP groups was 0.00 (effect size $g=0.00$), and the 95% confidence interval was −0.18 to 0.18 (effect size confidence interval $g=-0.74$ to 0.74). The observed group mean difference between the RM and NRM groups was 0.03 (effect size $g=0.15$), which was in the predicted direction, and the 95% confidence interval was −0.13 to 0.19 (effect size confidence interval $g=-0.60$ to 0.89). There were no significant group differences in any individual physiological response measure, or in IES-R change score. The observed difference in IES-R change score group means between the RM and PP groups was −4.2 (effect size $g=-0.27$), which was in the *non*-predicted direction, and the 95% confidence interval was −16.4 to 8.0 (effect size confidence interval $g=-1.01$ to 0.49). The observed group mean difference between the RM and NRM groups was 5.3 (effect size $g=0.44$), which was in the predicted direction, and the 95% confidence interval was −4.0 to 14.6 (effect size confidence interval $g=-0.32$ to 1.18). Note that the above confidence limits in the predicted direction for PPrb and IES-R change score were large enough so that failure to find the hypothesized effect of reactivation mifepristone might have represented a Type II error.

3.3.3. Additional analyses

When gender was added as a factor to the ANOVA, there were no significant gender or group (i.e., drug condition) main effects, or gender × group interaction on PPrb score or IES-R change score. At the time of the script-driven imagery procedure on Day 8, nine subjects were found to have positive urine drug screens for one or more potentially confounding substances, including opiates, barbiturates, cocaine, and THC. When the analyses were repeated excluding these subjects, the group differences for PPrb and IES-R change scores remained non-significant.

3.4. Discussion

The results of Study Two failed to show significant differences among reactivation mifepristone, non-reactivation mifepristone, and placebo subjects. The inclusion of a control group that received placebo on Day 0 and Day 2 rules out the potential confounding role of non-specific drug effects, which limited the interpretation of the negative results with propranolol in Study One. The dose of mifepristone given, i.e., 1800 mg, is greater than four times the dose shown to induce inhibition of GR receptors (Bertagna et al., 1984), suggesting that the lack of effect shown was not due to inadequate dosage.

Mifepristone has anti-progesterone effects in addition to anti-glucocorticoid effects. Given the different concentrations of sex hormones as a function of sex and the cross-talk between the hypothalamic–pituitary–adrenal and hypothalamic–pituitary–gonadal axes (Viau, 2002), it is possible that men and women would show different responses to mifepristone administration. However, we found no significant difference in the effect of mifepristone on physiological reactivity or change in PTSD symptoms between genders. Conclusive interpretations are limited, however, by a small sample size of women (RM 3, NRM 5, PP 2).

4. Study Three

4.1. Introduction

As discussed above, successful pharmacological blockade of memory reconsolidation depends upon two steps. First the memory must be destabilized by its reactivation (retrieval). Second, the drug must interfere with the reconsolidation of the reactivated memory. The absence of a reconsolidation blockade effect in Studies One and Two may have resulted from failure to destabilize the memory in the first place, rather than pharmacological inadequacy of the reconsolidation blocker. Results of a recent study in animals suggest that memory traces that are formed under highly stressful conditions resist destabilization and thus may be inaccessible to reconsolidation blockers (Bustos et al., 2010). In the cited study, however, when memory reactivation was preceded by the administration of D-cycloserine (DCS), subsequent reconsolidation blockade by midazolam then became successful, suggesting that DCS may promote the destabilization of resistant memory traces (in addition to its better recognized role in strengthening extinction learning). DCS acts as a partial agonist at brain N-methyl-D-aspartate (NMDA) receptors, which have been implicated in memory destabilization in animals (Ben Mamou et al., 2006). The traumatic memories of individuals with PTSD have by definition been formed under highly stressful conditions and thus may resist destabilization. We hypothesized that individuals with PTSD who underwent memory reactivation that was preceded first by DCS (the putative memory destabilizer) and second by mifepristone (the putative reconsolidation blocker) (RMD group) would show smaller physiological responses during script-driven imagery testing a week later compared to those who received two placebos (PL).

4.2. Methods

4.2.1. Subjects

4.2.1.1. Recruitment and inclusion criteria. Research subjects were males and females ages 18–62, drawn from advertisements in the local Boston media, who met diagnostic criteria for PTSD.

4.2.1.2. Exclusion criteria. In addition to the exclusion criteria listed in Study Two, subjects were excluded if they had a condition that contraindicated the administration of DCS such as hypersensitivity to cycloserine, epilepsy, renal insufficiency, systolic blood pressure greater than 180 or less than 100, Meniere's disease, or migraine.

4.2.1.3. Ethical approval and informed consent. After a full explanation of the study procedures, which had been approved by the Partners Human Research Committee and the U.S. Army Medical Research and Materiel Command Human Research Protection Office, subjects gave written informed consent prior to participation.

4.2.2. Study medication

DCS is approved for use as an anti-tuberculosis antibiotic and for treatment of urinary tract infections. Its use in Study Three was approved by the FDA under the same IND as in Study Two. DCS reaches peak blood levels 4–8 h after oral administration. On Day 7, we administered either 100 mg oral DCS followed by 1800 mg oral mifepristone, or two placebos. The DCS dose was in the range of that used in other studies of PTSD and anxiety disorders (albeit in different designs; e.g., see Difede et al. (2014)).

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A double-blind 1:1 randomization schedule, stratified by gender, was utilized. The study medications were well-tolerated by all subjects with few reported side effects.

4.2.3. Equipment and physiological measures

See Section 2.

4.2.4. Procedures

On Day 0, a psychologist administered the CAPS and SCID. Urine samples were collected and analyzed for substances of abuse. On Day 7, female subjects of child-bearing potential underwent a serum pregnancy test; none were found to be pregnant. Subjects randomized to the RMD group received DCS approximately 4 h prior to mifepristone administration. Mifepristone was then administered 90 min prior to traumatic memory retrieval via the script preparation procedure. Subjects randomized to the PL group received matching placebo capsules at each time point. Subjects also completed IES-R as above. On Day 14, subjects underwent the script-driven imagery session. Urine samples were again collected and analyzed for substances of abuse.

4.2.5. Data reduction and statistical analysis

See Section 2. Between-group Student's *t*-tests were performed for all outcome measures. Two-way ANOVA was performed to incorporate gender into the analyses.

4.3. Results

4.3.1. Subject randomization and characteristics

Two subjects randomized to RMD and one subject randomized to PL did not meet PTSD diagnostic criteria as determined by the CAPS. Data from these three subjects were excluded from the analysis, leaving final group sizes of RMD $n=16$ (nine female) and PL $n=15$ (eight female).

As shown in the top panel of Table 3, there were no significant group differences in age, baseline IES-R score, or CAPS score. Current comorbid mental disorders according to the SCID included, in the RMD group: panic disorder ($n=3$), dysthymia ($n=2$), MDD ($n=2$), OCD ($n=2$), simple phobia ($n=2$), social phobia ($n=2$), bipolar II ($n=1$), eating disorder ($n=1$), GAD ($n=1$); and in the PL group: dysthymia ($n=3$), MDD ($n=3$), simple phobia ($n=2$), social phobia ($n=3$), OCD ($n=2$), panic disorder ($n=2$), GAD ($n=1$). Traumatic events were as follows: in the RMD group, rape or sexual violence ($n=7$), actual or threatened serious injury to self ($n=5$), witnessing death of others ($n=3$), vehicular homicide ($n=1$); and in the PL group, rape or sexual violence ($n=6$), actual or threatened serious injury to self ($n=6$), witnessing death of others ($n=1$), learning of serious injury to loved one ($n=1$), combat ($n=1$).

4.3.2. Outcome measures

As shown in Table 3, the group difference in Day 14 PPrb score was not significant. Specifically, the observed difference in group means was −0.01 (effect size $g=-0.04$), which was in the *non*-predicted direction, and the 95% confidence interval was −0.18 to 0.16 (effect size confidence interval $g=-0.75$ to 0.66). There were no significant group differences on any individual physiological response measure or in IES-R change score. The observed difference in IES-R change score group means was 3.9 (effect size $g=0.27$), which was in the predicted direction; the 95% confidence interval was −6.9 to 14.7 (effect size confidence interval $g=-0.46$ to 0.98). Note that these confidence limits in the predicted direction for PPrb and IES-R change score were large enough so that failure to find the hypothesized effect of DCS plus reactivation mifepristone might have represented a Type II error.

4.3.3. Additional analyses

A two-way ANOVA yielded a main effect of gender on PPrb score, $F(1,27)=5.25$ ($p=0.03$), with females showing higher overall reactivity. However there was no significant main effect of group (i.e., drug condition), or significant gender × group interaction. There was no significant main effect of gender, group, or significant gender × group interaction on IES-R change score.

According to urine testing on Day 14, one subject was found to be taking a potentially confounding substance at the time of the script-driven imagery procedure. Excluding this subject from the analyses did not change the findings presented above.

4.4. Discussion

The results of Study Three revealed no significant difference between the group receiving DCS plus mifepristone and the placebo control group. We had hoped to enable mifepristone-induced reconsolidation blockade by promoting traumatic memory destabilization with DCS, but according to the present results, this goal was not achieved.

Women showed significantly higher overall levels of physiological reactivity. However, there was no significant difference in the drug effect between genders. Although the Study Three samples were stratified by gender, no information on menstrual cycle was collected. Given the effects of mifepristone on the progesterone system and the possible interactions among menstrual cycle, progesterone, and traumatic memories (Bryant et al., 2011), analysis according to menstrual phase may be prudent in future studies. Other pharmacological agents that target the glucocorticoid system alone, such as metyrapone (Marin et al., 2011), may be suitable in future studies as well.

5. General discussion

Study One aimed to replicate and extend earlier findings that propranolol accompanying traumatic memory reactivation weakens physiological responding during subsequent mental imagery of the traumatic event (Brunet et al., 2008). Studies Two and Three pursued novel pharmacological interventions for traumatic memory reconsolidation blockade, specifically mifepristone alone or in combination with DCS. Unfortunately, the results of all three studies failed to show significant effects of pharmacological agents administered prior to traumatic memory retrieval on subsequent physiological responses during script-driven traumatic imagery, or on change in PTSD symptoms assessed by the Impact of Event Scale (IES-R). However confidence interval analyses indicated that we cannot entirely rule out the possibility of Type II error having played a role in the negative results in Studies Two and Three.

Failure to find significant differences between groups in these studies may reflect an insensitivity of the outcome measures. Although heart rate, skin conductance, and electromyogram responses have been found able to identify individuals with versus without PTSD during script-driven traumatic imagery, their sensitivity is only fair (Orr et al., 2002). They may not always be able to detect changes induced by a single dose of medication. The three studies also failed to find pharmacological effects on self-reported PTSD symptoms quantified by the IES-R. However, it may be unrealistic to expect a therapeutic effect of a single session of memory reactivation plus drug.

Another possible explanation for the negative results could be a floor effect. The average PPrb scores in the control groups across the three studies here ranged from 0.32 to only 0.44, meaning that the average PTSD control subject had less than a 50% likelihood of being psychophysiologically classified as having PTSD. These PPrb scores are substantially lower than we have previously seen in

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persons with PTSD (Orr et al., 2012). The average CAPS scores across the three studies ranged from 59 to 67, which is consistent with only mild to moderate PTSD (Weathers et al., 2001). Hence our subjects may not have had severe enough PTSD for us to be able to detect an effect of the drug interventions. The recruitment of quality research subjects is an ongoing difficulty faced in clinical research. Individuals recruited from the community, with the incentive of a participation fee, differ from a treatment-seeking population. Persons with the most severe PTSD may be hesitant to volunteer for research studies. In Study Three, women showed significantly higher reactivity than men despite no difference in CAPS scores. Given that the prevalence of PTSD is also higher in women than in men (Tolin and Foa, 2006), perhaps a female population would be a more suitable target for future studies of reconsolidation blockade in PTSD.

The tests of the three studies' hypotheses consisted of cross-sectional comparisons of physiological reactivity between subject groups. Baseline physiological reactivity was not assessed in these studies out of a fear of habituating the subject to the script-driven imagery procedure. However, it is possible that a repeated-measures design that measured changes in physiological reactivity both before and after the interventions could have been more sensitive to the hypothesized effects. Additionally, we did not obtain physiological measures during the preparation of the traumatic scripts. Such data might have provided a validity check on the strength of memory retrieval at the time, and the resulting degree of putative memory destabilization. These design modifications should be considered in future studies. Baseline physiological testing might also be used to select those individuals who show heightened reactivity, so as to avoid potential floor effects and target those individuals with more severe PTSD.

It is possible that the script-driven imagery procedure is sometimes insufficient to induce traumatic memory destabilization (for possible reasons, see Sevenster et al. (2012) and Soeter and Kindt (2013)), even when it is preceded by the administration of DCS. The choice to give the candidate reconsolidation blockers before memory retrieval was dictated by the consideration that oral propranolol and mifepristone take approximately 90 min to reach peak plasma levels in the human body. Because reconsolidation begins only a few minutes after memory reactivation, post-reactivation administration of these drugs may produce negative results because there will not be sufficient time for their effect to be exerted before a substantial degree of reconsolidation has already occurred. However, having selected this design, we cannot rule out the possibility that the propranolol or mifepristone given in advance may have attenuated memory reactivation during script preparation and thereby failed to produce destabilization of the traumatic memories. We have discussed this issue in greater detail in Brunet et al. (2011). The present results illustrate that translating reconsolidation blockade into clinical applications is unlikely to be simple or straightforward. More research is needed to search for potent pharmacological or other neurotherapeutic agents and administration paradigms that might confer lasting clinical benefit in PTSD by modifying or weakening the underlying traumatic memory, and optimal designs in which to test them.

Conflict of interest

None of the authors have competing interests.

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Randomized Placebo-Controlled Trial of Propranolol Plus Trauma Memory Reactivation for PTSD

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Abstract

We performed a randomized, double-blind, placebo-controlled trial of the efficacy of the **β**-blocker propranolol, administered prior to trauma memory reactivation, in reducing symptoms in subjects with chronic posttraumatic stress disorder (PTSD). During each of six weekly treatment sessions, subjects (Ss) received 1.0 mg/Kg oral propranolol or placebo 60-min. prior to 10 min. of imaginal exposure to the traumatic event prompted by reading a prepared script. Propranolol Ss showed a steeper decline in weekly PTSD Checklist scores. Pre-to post-treatment reduction in Clinician-Administered PTSD Scale scores was greater in the propranolol group.

Introduction

Reactivating a consolidated memory through retrieval may return it to an unstable state from which it must be reconsolidated if it is to persist.[1] The **β**-adrenergic blocker propranolol may oppose reconsolidation in humans.[2,3] Blockade of reconsolidation has been suggested as a potential novel treatment for PTSD.[1] Propranolol administered at the time of trauma memory reactivation has been found to reduce subsequent physiological responding during trauma imagery.[4] A series of six weekly open-label propranolol plus trauma memory reactivation trials was found to reduce PTSD symptoms.[5] Here we performed a randomized, double-blind, placebo-controlled trial of propranolol plus reactivation in an attempt to reduce symptoms in subjects with chronic PTSD.

Methods

Subjects (Ss) comprised a convenience sample of persons ages 18-65 with chronic PTSD. Ss with asthma or heart disease were excluded. Thirty Ss (20F, 10M) randomized to propranolol (PROP) presented for the first treatment session; mean age=36.2 (SD=9.6); mean education=14.6 (SD=3.1); 21 completed treatment and underwent the post-treatment assessment. Twenty-three Ss (12F, 11M, group difference p=0.40) randomized to placebo (PLA) presented for the first treatment session; mean age=43.7 (SD=11.0), p=0.01; mean education=15.3 (SD=3.2), p=ns; 20 completed treatment and underwent the post-treatment assessment.

Instruments included the PTSD Checklist-Specific Version (PCL) and the Clinician-Administered PTSD Scale (CAPS). The PCL was administered prior to each treatment session and at the post-treatment assessment with reference to the preceding week. The CAPS was administered at one-week pre- and post-treatment.

Procedure. At the pre-treatment assessment, a one-page "script" of the S's event that caused the PTSD was prepared. A week later, there began six weekly treatment sessions. At each session, the S received 1 mg/Kg short-acting oral PROP or PLA (same for each session), waited 60-min., read the script aloud to an investigator and then engaged in mental imagery of the personal traumatic event the script portrayed for 10-min.

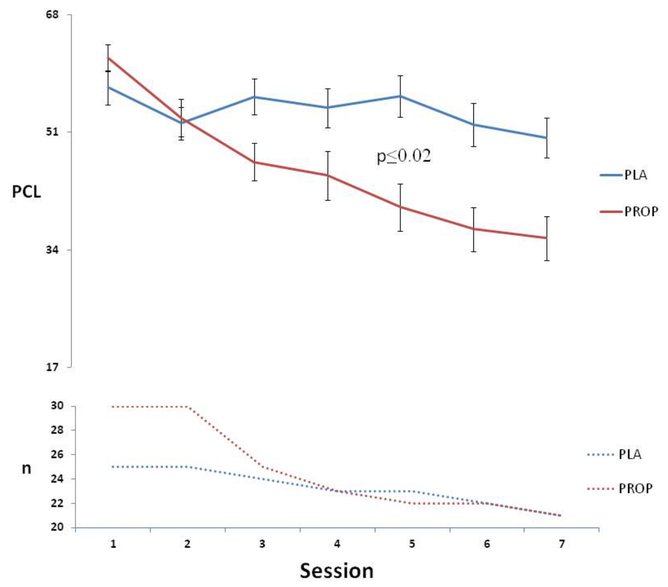

Figure 1. Top: Weekly PTSD Checklist (PCL) scores. Bottom: Number of subjects who completed the PCL.

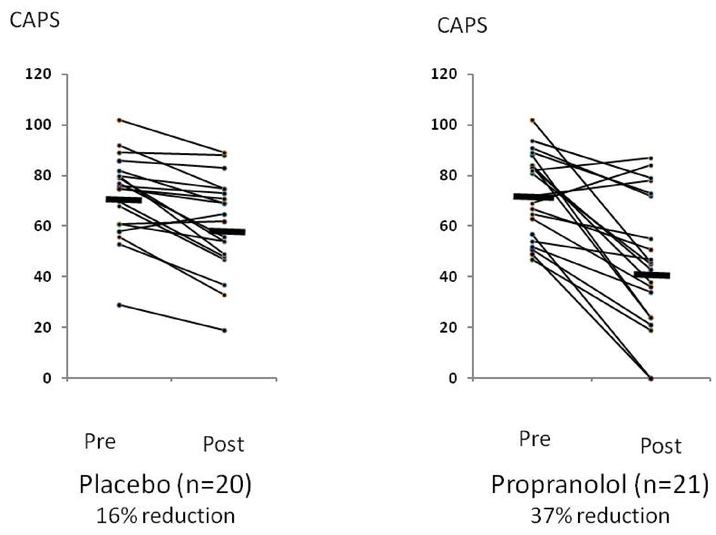

Figure 2. Pre- and post-treatment Clinician-Administered PTSD Scale (CAPS) scores in completers. Heavy bars indicate means.

Results

Weekly PCL scores are shown in Figure 1. Beginning with treatment Session 3, and continuing until the post-treatment assessment (designated Session 7), PCL scores were significantly lower ($p \leq 0.02$) in subjects who were receiving weekly propranolol than in subjects who were receiving placebo.

Pre-and post-treatment CAPS scores in the 21 PROP and 20 PLA Ss who completed all six treatment sessions appear in Figure 2. Change scores were subjected to two-factor (Gender, Drug) analysis of covariance (ANCOVA) with age as a covariate. Neither the Gender main effect nor the Gender x Drug interaction was statistically significant. The Drug main effect yielded $F(1,37)=3.4$, $p<0.05$.

Collapsed across Gender, within-group pre- to post-treatment effect sizes, calculated as decrease in CAPS scores divided by pre-treatment standard deviation, were as follows: propranolol group 1.6, placebo group 0.7.

In an intent-to-treat sensitivity analysis, each subject with a missing post-treatment CAPS score was assigned the mean CAPS change score of the *placebo* group. Applying the same ANCOVA to these data, the Drug main effect remained significant: $F(1,49)=2.8$, $p<0.05$. Too few subjects returned for a scheduled 6-month assessment to permit data analysis.

Conclusion

The results indicate that a series of weekly, brief, imaginal exposures to the traumatic event were more effective in reducing PTSD symptoms when these exposures were preceded by propranolol than by placebo. The within-group effect size for reduction in total CAPS score of 1.6 compares favorably with the effect sizes reported for the current treatment of choice for PTSD, viz., cognitive behavior therapy (CBT). Yet the duration of imaginal exposure to the traumatic event in the present study was less than one-tenth that required by CBT. It is plausible that the superior therapeutic results achieved in the propranolol group were due to blockade of reconsolidation of the trauma memory that was activated by the imaginal exposure. However, further studies that include appropriate controls will be required to establish this, including administration of drug in the absence of reactivation, measurement of symptoms a few hours following the exposure, and long-term follow-up in an adequate sample to permit the evaluation of spontaneous recovery of PTSD symptoms.

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Notes

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2. The use of propranolol for this indication is off-label.
3. The authors have no conflicts of interest to disclose.
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